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# **Development of Colonic Fermentation in Early Life**

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## SUMMARY

Carbohydrates that escape digestion and absorption in the small intestine enter the colon where they are anaerobically metabolised by the colonic bacteria. Colonic fermentation of carbohydrates produces short chain fatty acids (SCFA) (Cummings and Englyst, 1987) which are rapidly absorbed which salvages energy and prevents osmotic water loss (Kien *et al.*, 1990).

Bacterial colonic fermentation has been well investigated in adults but our knowledge in infants is limited. Neonatal diet and the weaning period are thought to be critical factors to the establishment of the intestinal microflora. The early events in bacterial colonisation of the gut may be critical in establishing the health of the infant as well as determining the properties of the adult flora.

It is well established that the flora of breast-fed and formula-fed infants differs (Balmer and Wharton, 1989) and this is reflected in the profile of faecal SCFA (Edwards *et al.*, 1994). However there is no information on the flora and faecal SCFA of infants that receive a mixture of breast milk and formula milk before weaning. As more mothers begin breast-feeding and then do not continue, this 'mixed fed' group are increasingly important in the UK. Little is known about the factors which change the colonic microflora at weaning when infants are exposed to a wide range of new substrates. If substrates are undigested and unfermented these substrates will pass through the colon unmetabolised causing increased faecal output and possible diarrhoea.

Very few studies have investigated the effect of weaning on the faecal flora. Many weaning foods will contain dietary fibre and other complex carbohydrates and it is important to know how easily these will be fermented and the effect these substrates will have on the development of the colonic microflora.

We wished to test the hypothesis that neonatal feeding practice determines the rate and pattern of development of the colonic microflora during weaning.

After consideration of fermentation models an *in vitro* fermentation model (modified from Adiotomre *et al.*, 1990) was chosen to determine the ability of infants to ferment a range of simple and complex carbohydrates.

Breast-fed and formula-fed infants were investigated in a cross-sectional pilot study. Faecal samples were collected from infants at different stages during weaning. The cross-sectional study demonstrated that there were differences in the fermentation capacity of breast-fed and formula-fed infants. In formula-fed infants there was no difference between pre-, early and late weaning for any substrate. In breast-fed infants the ability to ferment raftilose increased at early weaning and the ability to ferment soyabean polysaccharide increased at late weaning. In both groups cultures containing soyabean polysaccharide, produced lower total SCFA than adults, even at late weaning. The pilot study indicated the need for a larger number of infants that would be followed longitudinally in order to draw definitive conclusions.

The pilot study allowed us to design the longitudinal study which investigated the relative fermentation capacity for a wider range of carbohydrates. Results of the longitudinal study confirmed those of the cross-sectional study. All infant feeding groups were equally able to ferment the simple sugars and oligosaccharides at all development stages. There were significant increases in total SCFA produced in cultures containing the complex carbohydrates at late and very late weaning breast-fed infants. In formula-fed infants no differences were seen between any development stage for any substrate, apart from an increase in total SCFA produced between pre- and late weaning. In mixed fed infants total SCFA produced in cultures containing pectin increased significantly at late weaning. With the other complex carbohydrates total SCFA produced increased at early weaning from pre-weaning. In all infant feeding groups, cultures containing complex carbohydrates produced lower levels of total SCFA than adults.

Breast-fed infants had SCFA profiles of acetate and lactate with cultures containing simple sugars before weaning. In contrast, raftilose™ and complex carbohydrate produced predominantly acetate and propionate before weaning. Both propionate and butyrate increased for all substrates at late and very late weaning and no lactate was produced. In formula-fed and mixed fed infants mainly acetate and propionate were produced with some butyrate at pre- and early weaning. At late and very late weaning proportions of butyrate increased significantly. In mixed fed infants there was also a decrease in propionate at late and very late weaning in cultures containing lactose, raftilose™ and pectin.

Investigations of faecal SCFA confirmed previous findings. Total SCFA increased between pre- and late weaning in breast-fed infants and pre- and very late weaning in mixed fed infants. In contrast, no differences were seen in formula-fed infants between any stage of weaning. Proportions of propionate and butyrate increased significantly and lactate decreased throughout weaning in breast-fed infants. Fewer changes were seen in formula-fed infants although there was a significant increase in butyrate at the later weaning stages from early weaning. Similarly in mixed fed infants there was a significant increase in butyrate throughout the weaning stages.

Excretion of faecal starch has been seen in infants up to 3 years of age (Verity and Edwards, 1994). Faecal starch increased significantly from pre-weaning in breast-fed and formula-fed infants. In mixed fed infants there was an increase in faecal starch between early and late weaning although this did not reach significance but at very late weaning faecal starch significantly decreased. This suggests maturation of gut function and development of the colonic bacteria enabling better digestion and fermentation of starch in all infants. At early weaning breast-fed and mixed fed infants excreted more faecal starch than formula-fed infants. This may be an indication that the development of the colonic flora is slower in breast-fed and mixed fed infants than formula-fed infants.

Excretion of faecal fat in breast-fed infants was significantly lower at pre- and early weaning than in formula-fed infants. This can be correlated with better mechanisms for digestion of fat in breast-fed infants. By late weaning were no differences between the two feeding groups.

Percentage daily energy lost through excretion of fat and starch was estimated to be negligible.

The present study represents a detailed longitudinal investigation of fermentation capacity in breast-fed, formula-fed and mixed fed infants. Results suggest a slow maturation of the colonic flora and its fermentation capacity that may allow manipulation for future health benefits. In addition, distinct differences exist between the three infant feeding groups which is probably related to the composition and development of the bacterial flora.

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# **Chapter 1**

## **The Development of the Colonic Microflora in Infancy**

## **1.1 INTRODUCTION**

The human colon has a major role in salvaging energy and nutrients that escape digestion and absorption in the small intestine (Lifschitz, 1996). These undigested and unabsorbed nutrients are anaerobically metabolised, producing short chain fatty acids (SCFA) and gas (Cummings and Englyst, 1987). SCFA are rapidly absorbed (McNeil *et al.*, 1978) and provide energy for the colonic mucosa and for the body as a whole (Cummings, 1981). The ability to ferment different carbohydrates has been extensively investigated in adults (Englyst *et al.*, 1987; McBurney and Thompson, 1987; Adiotomre *et al.*, 1990; Vince *et al.*, 1990) but few studies have concentrated on infants. The salvage mechanism is dependent on the colonic bacterial microflora which develops in the first few weeks of life and is heavily influenced by the initial feeding method (breast or formula feeding) (Balmer and Wharton, 1989; Edwards *et al.*, 1994). This colonic function is of particular importance in the first year of life when pancreatic and digestive function is immature (Hamosh, 1996). The immune system is also immature at birth and its correct maturation (avoidance of allergies) is determined by interactions with the gut microflora (Helgeland and Brandtzaeg, 2000). The colonisation of the intestine in the first year of life may also determine the properties of the final adult microflora, which could have major health implications. Thus it is essential that we have a good understanding of the bacterial colonisation of the infant gut and the maturation of the metabolic capabilities of the resultant flora.

Before considering the development of the infant colonic microflora it is important to understand the composition and metabolic activities of the indigenous intestinal flora of the adult.

## **1.2 ADULT COLONIC MICROFLORA**

The composition of the colonic flora of the adult human has been extensively investigated (Moore and Holdeman, 1974; Savage, 1977; Finegold and Sutter,

1978; Moore *et al.*, 1978; Croucher *et al.*, 1983; Wilson and Blichington, 1996, Hopkins *et al.*, 2001). It is very difficult to obtain accurate data on the bacterial flora of the human colon, especially of the flora in the proximal colon where most fermentation occurs. It is almost impossible to obtain samples from the proximal colon without altering the flora (see below). Moreover, the standard cultivation and identification methodologies have been primarily developed for isolation and identification of pathogens and may not be sufficient for the complex normal flora (Hill, 1995). These difficulties have resulted, in the main, in assumptions about the colonic flora being inferred from the composition of the normal faecal flora and even that data is limited.

### **1.2.1 Methods for characterisation of the flora**

Traditional methods for characterising the flora of the gut have relied on simple enumeration of species using selective culture, isolation and characteristic biochemical properties (O'Sullivan, 1999). However, recent studies have indicated that a significant amount of human faecal flora is non-culturable and adequate methods to determine the colonic flora are still lacking. Culture-based studies are still considered to be the 'gold standard' (Vaughan *et al.*, 2000) but have drawbacks. Prior to the culture procedure, there is the problem of obtaining a sample. Using faeces is the easiest method of obtaining a sample, but this only represents the rectum and rectosigmoid region (Hill, 1995). The inaccessibility of the colon makes direct sampling difficult. Samples of colonic luminal contents can be obtained at surgery but generally the bowel has been emptied prior to the operation. A rigid proctoscope or flexible sigmoidoscope may be used. However, both these methods obtain samples from lower in the colon. Sampling from higher in the colon can be achieved using colonoscopy, but again bowel cleansing is required thereby rendering the sample unrepresentative. Mucosal associated flora can be sampled by mucosal biopsy but this method is not accurately quantitative. Another approach is to give the subject a capsule to swallow (Drasar and Hill, 1974). This capsule can be opened and closed by remote control to

allow sampling. A problem with this method is that it may allow bacteria to multiply during transit through the colon and this would invalidate the results.

Once the sample has been obtained, it is necessary to process it quickly. Enzyme activities and exposure to oxygen decrease the viability of the bacteria. It may be necessary to transport the sample to the laboratory in an anaerobic tube. Freezing samples at -40°C in a cryoprotective media allows faecal samples to be stored (Crowther, 1971). The impact on the full characterisation of the flora, however, should be checked, using more modern culture techniques, as there may be selective loss of individual species.

The basic division of bacterial groups is based on Gram staining properties, which relate to the permeability of the bacterial cell wall to dyes. The further division into species and subspecies is dependent on cultivation and isolation of individual bacteria. Problems of culturing include both the choice of culture media and cultivation environment. Because of the strictly anaerobic nature of most organisms, cultivation has to take place in an anaerobic environment. The agar plates used need to have been deoxygenated (pre-reduced) before plating out in an anaerobic cabinet and then finally incubated in a suitable anaerobic environment. Alternatively the Hungate roll-tube method (Hungate, 1966) can be used where inoculation of diluted faecal sample is in pre-reduced agar roll tubes and then the tubes are incubated in a self-contained oxygen-free atmosphere. Both enriched media and selective media should be used to ensure detection of organisms present in low numbers. Selective media may inhibit the growth of even those organisms for which they are designed, and may therefore underestimate true populations. Moreover, selective media are still not available for many of the strict anaerobes.

Following isolation, identification of individual species has been based on phenotypic profiles such as carbohydrate fermentation profiles, metabolic end product analysis, antibiotic and dye resistance along with enzymic activity tests (Holdeman *et al.*, 1977; Summanen *et al.*, 1993). These techniques are not only

laborious, but also slow growth means that the identification procedure can take several days. Indeed these assays and the gram staining procedure may not be the most appropriate divisions and categorisation of individual bacteria groups in light of the new information being provided by comparisons of bacterial RNA and DNA.

Molecular tools such, as hybridisation probes, based on ribosomal RNA sequences, may be the way forward. There are several new and novel methods available, Table 1.1, (Vaughan *et al* 2000). Complex microbial communities should be described more accurately in terms of diversity, structure and dynamics. As with traditional culturing techniques, there are several problems with these new techniques. One of the main problems is the extraction and purification of DNA and/ or RNA from the sample. Numerous methods, that use enzymatic, chemical and mechanical breaking of the cells, have been reported for extraction of nucleic acids from faecal samples (Zoetendal *et al.*, 1999; Wang *et al.*, 1996; Klijn *et al.*, 1995). A method of disruption of bacteria by bead beating (glass or zirconium beads added to sample in buffer and shaken vigorously) has been recently described. This can be used alone or in conjunction with other methods (Dore *et al.*, 1998). Bead beating may have the disadvantage of shearing the nucleic acids which leads to low yields and may also increase chimeric structures during amplification. The highly conserved regions of rRNA molecules can serve as target sequences for universal or domain probes. The intermediate and more variable regions are appropriate for the determination of genus, species and sometimes strain specific hybridisation probes (Dore *et al.*, 1998).

Another technique is fluorescent in situ hybridisation (FISH) using ribosomal RNA oligonucleotide probes (Harmsen *et al.*, 2000). This system is automated, and digital pictures obtained by epifluorescent microscopy can be analysed by computer. Individual species can be identified in a sample in situ, i.e., in a culture sample of intestinal contents or biopsy of gut wall. Other techniques including denaturing or temperature gradient gel electrophoresis (DGGE/ TGGE), single-strand conformation polymorphism (SSCP) and terminal-restriction fragment

**Table 1.1 Advantages and disadvantages of microbial identification methods**

<b>Method</b>	<b>Advantages and Disadvantages</b>
<b><i>Direct microscopic count</i></b> (Holdeman and Moore, 1974)	Total count of all living and dead micro-organisms
<b><i>Viable count, aerobic incubation</i></b> (Brown, 1977)	Selective agars enable identification down to family and genus level. Aerobic count is <1% of anaerobic viable count.
<b><i>Viable count, anaerobic methods</i></b> (Drasar, 1967)	Counts of oxygen intolerant anaerobes. Best recovery with anaerobic cabinet rather than anaerobic jar.
<b><i>Hungate roll-tube method</i></b> (Hungate, 1966)	'Gold standard'. Can be used to culture even very oxygen sensitive anaerobes. May not be representative. Slow.
<b><i>16S rDNA (deoxyribonucleic acid) sequencing</i></b> (Wilson and Blichington, 1996)	Used for identification. Large scale cloning is arduous.
<b><i>DGGE / TGGE (denaturing / temperature gradient gel electrophoresis)</i></b> (Zoetendal <i>et al.</i> , 2000)	Used for identification and rapid detection of specific groups. Semi-quantitative
<b><i>T-RFLP (terminal-restriction fragment length polymorphism)</i></b> (Marsh, 1999)	Used for identification. Sensitive and rapid. Semi-quantitative. A clone library is necessary.
<b><i>SSCP (single strand confirmation polymorphism)</i></b> (Schwierger and Tebbe, 1998)	Rapid. Semi-quantitative. A clone library is necessary.
<b><i>Dot-blot hybridisation</i></b> (Dore <i>et al.</i> , 1998)	Enables detection and enumeration. Requires probe design. Laborious and throughput slow.
<b><i>FISH (Fluorescent in situ hybridisation)</i></b> (Franks <i>et al.</i> , 1998)	Enables detection and estimates relative abundance. Requires probe design. Can be laborious and throughput slow.
<b><i>FISH/ Flow cytometry</i></b> (Jansen <i>et al.</i> , 1999)	Allows enumeration. Potential for high throughput but method still developing.

length polymorphism (T-RFLP) (Zoetendal *et al.*, 1998; Schwierger and Tebbe, 1998; Marsh, 1999) have been shown to be useful tools in analysis of complex microbial communities. These techniques provide idiosyncratic gel patterns similar to human DNA fingerprints. Although these methods give consistent results and are able to identify non-culturable flora, at present, the cost can be prohibitive and the process slow in comparison to traditional methods. The advantages and disadvantages of culture and molecular techniques are shown in Table 1.1.

### **1.2.2 Composition of the adult microflora**

The colonic flora of the adult has been the subject of many studies (Moore and Holdeman, 1974; Savage, 1977; Finegold and Sutter, 1978; Moore *et al.*, 1978; Croucher *et al.*, 1983). Prior to the 1960s descriptions of the intestinal microflora were very simple. In the late '60s improvements were made for cultivating bacteria such as the Hungate roll-tube method (Hungate, 1966; Holdeman and Moore, 1972) that allowed an improved knowledge of human colonic microflora. Studies over the last thirty years have shown the diversity of the adult colonic flora. As discussed above, the new molecular techniques have indicated even greater complexity.

Average faecal bacterial counts are greater than  $10^{11}$  colonies per gram and there are at least 400 species (Moore and Holdeman, 1974). The new techniques, which measure rRNA or DNA (Dore *et al.*, 1998; Harmsen *et al.*, 1999; Vaughan *et al.*, 2000), however, suggest that up to 80% of the bacterial RNA in faeces is not accounted for by known bacteria. The number of species, therefore, may be even higher.

Most of the bacteria are strict anaerobes, outnumbering facultative organisms by 1000 to 1 (Evaldson, 1982). The normal faecal flora (Table 1.2) is dominated by strictly anaerobic gram-positive and negative rods, which represent 99% of the total flora. Other anaerobes present in smaller numbers are fusobacteria,



veillonella (gram-negative cocci), peptococci and peptostreptococci (both gram-positive cocci) and clostridia (gram-positive bacilli).

The microaerophilic lactobacilli and streptococci are at an intermediate level but still outnumber the dominant facultative organisms, mostly the coliform group. The counts of the dominant organisms are stable but those of the minor organisms show more fluctuation (Hill, 1995).

**Table 1.2      The normal faecal flora of healthy adult humans as detailed using conventional culture techniques**

	Dominant species	Log <sub>10</sub> bacteria per gram faeces
<b>Non-sporing anaerobes</b>		
Bacteroides	<i>B.fragilis</i> , <i>B.vulgatus</i> , <i>B. thetaiotaomicron</i>	10-11
Bifidobacterium	<i>B.adolescenti</i> , <i>B. bifidum</i> , <i>B. infantis</i>	10-11
Eubacterium.	<i>E. aerofaciens</i>	9-11
Propionibacterium		9-11
Veillonella.		5-8
<b>Sporing Anaerobes</b>		
Clostridium spp.	<i>C. perfringens</i>	5-9
<b>Sporing aerobes</b>		
Bacillus.		4-6
<b>Microaerophiles</b>		
Lactobacillus	<i>L. acidophilus</i> , <i>L. brevis</i>	7-9
Streptococcus	<i>S. hansenii</i>	7-9
Enterococci		5-7
<b>Facultative organisms</b>		
Coliforms		7-9
Other Enterobacteria	<i>E.coli</i> , <i>Klebsiella</i>	5-9

Adapted from Hill, 1995, Finegold *et al.*, 1983

### 1.2.3 Factors affecting the microflora of the adult

There are many factors controlling the gut microflora in the upper and lower intestine (Table 1.3). Lysozyme, bile and pancreatic secretions and immune systems may all inhibit some of the flora (Kleessen *et al.*, 2000). However, other studies have not shown evidence of these effects (Savage, 1977). Conditions in the large intestine such as the stagnant motility (Cummings, 1978) allow a much more luxuriant microbial growth (Table 1.4).

A variety of factors control the bacterial populations of the gut (Table 1.3). These may be related to the host, the bacteria and the environment. In order for bacteria to become established in the colon, their multiplication rate must be greater than their rate of elimination.

**Table 1.3 Factors controlling the gut flora composition**

<b>Physicochemical factors</b>	Intestinal pH
	Oxidation-reduction potential
	Nutrient supply
<b>Host-bacteria interactions</b>	Saliva
	Bile
	Gastric secretions
	Pancreatic secretions
	Immune systems
	Gut motility
<b>Microbe-microbe interactions</b>	Bacteriophages
	Bacteriocins
	Toxic metabolites
	Exogenous micro-organisms
	(pathogens, probiotics)
<b>Environmental factors</b>	Geographic location, climate
	Diet (drugs, antibiotics)
<b>Other factors</b>	Age
	Sex
	Stress
	Disease

Various studies have investigated the faecal flora of different populations with varied diets and different disease states (Drasar and Hill, 1974; Finegold *et al.*, 1974, 1975, 1977). Faecal flora studies of a number of populations show good similarity and a general agreement that the faecal flora of the large bowel is predominantly anaerobic. There were no major differences when different diets were compared, although individuals on chemically defined diets had a reduction in the number of faecal organisms (Attebery *et al.*, 1972). These studies have investigated different populations, such as Japanese and Western populations and strict vegetarians. However, other studies where the diet is changed by adding a quantity of a particular carbohydrate have demonstrated a change in the colonic microflora. For example, feeding oligosaccharides stimulated bifidobacteria growth (Gibson and Roberfroid 1995), and resistant starch may have similar effects (Silvi *et al.*, 1999). Use of these 'prebiotics' is a rapidly growing field. Prebiotics are non-viable food ingredients, which reach the colon where they are selectively fermented and increase the growth of probiotic organisms usually lactic acid bacteria. Prebiotics are thought to have various health benefits related to colonic cancer and immunological disease, but at present evidence is limited (Morgensen *et al.*, 2000). Probiotics, in contrast, are defined as live microbial food supplements which beneficially affect the host directly or indirectly by improving its microbial balance (Fuller, 1991). The alterations and improvements are mainly in the colon where they are thought to correct imbalances between the beneficial and harmful actions of bacteria (Pathmakanthan *et al.*, 2000). At present the most commonly used probiotics are from the genera *Lactobacilli* and *Bifidobacteria*.

The intestinal ecosystem of the normal adult is stable, although there are interindividual variations (Savage, 1977). The normal conditions of the colon are given in Table 1.4. These conditions may be further altered by the microflora itself to be more or less effective in influencing the microbial composition (Savage, 1977).

<b>Table 1.4                      Conditions in the human colon</b>	
<b>Factor</b>	<b>Colon</b>
Temperature	37- 39°C
pH	From 4.5 to alkaline
Stasis	Prolonged
Oxygen	Little
Oxidation reduction potential	Very low -400mV
Bile acids	Deconjugated, secondary
Mucin	May act as nutrient, Contributes to viscosity
Diet	May supply selective substrates for individual bacteria

It has been suggested that competition for nutrients is the most important factor controlling the number of total bacteria and the relative proportions of different species in the colon (Adlerberth, 1996). Nutrients are available from undigested and unabsorbed material from the small intestine but also from host secretions, such as mucins. The ability of an organism to compete more effectively for one of these carbohydrate sources will enhance its chances of survival.

The pH and redox potential of the germ-free colon are alkali and very oxygen rich, however, bacterial fermentation of carbohydrate (probably by facultative organisms in the first instance) reduces the pH to neutrality or slightly acid, and this also results in a negative redox potential and low oxygen potential. These conditions enable very oxygen sensitive anaerobes to grow. Once they are established, the facultative organisms compete poorly for nutrients hence, eventually, the anaerobes outnumber facultative organisms by 1000 to 1 (Evaldson, 1982).

Interbacterial interactions tend to control the composition of bacterial microflora rather than the total number. A wide range of bacterial species produce bacteriophage-virus particles (Hill, 1995). These are able to enter and lyse certain strains of the same species. Bacteriocins are also produced by many bacteria (Nomura, 1977). These antibiotic substances are lethal to a proportion of strains

of the same or related species. Bacterial metabolites may have antibacterial action against unrelated strains. Short chain fatty acids (SCFA) produced during fermentation of carbohydrate reduce the colonic pH. Many bacteria, for example, clostridia, grow less well in conditions of acidic pH whereas others such as lactobacilli and bifidobacteria prefer an acidic pH. SCFA have been shown to inhibit some bacteria themselves (Fay and Faires, 1975). SCFA production in the colon have been shown to prevent growth of salmonella species, although the low redox potential contributes to this effect (Cummings, 1981).

#### **1.2.4 Colonic microflora at different sites and ecological niches**

Some studies have attempted to quantify the flora at different sites in the colon. Non routine methods for collecting human colonic contents have included material collected post mortem after sudden death for direct analysis (Stephen *et al.*, 1987), or for supply of inoculum for *in vitro* studies (Macfarlane *et al.*, 1992). If the colonic material is obtained quickly (within four hours of death) this may give a true picture of the colonic flora. More invasive *in vivo* techniques that have been used include needle aspiration from patients during surgery, (Bentley *et al.*, 1972) and the swallowing of capsules that allow the remote control sampling of gastrointestinal tract contents (Pochart *et al.*, 1993). The invasive nature of these techniques, however, may lead to alterations in the flora.

The faecal flora is thought to reflect that of more proximal colon but there may be important differences. Bacterial counts differ throughout the large bowel (Drasar and Hill, 1974; Table 1.5). Although numerical differences were seen there did not appear to be significant qualitative differences in the flora. Bentley *et al.* (1972) studied 10 patients undergoing elective cholecystectomy and compared the microflora of the terminal ileum, caecum and transverse colon by direct needle aspiration with microbial counts in faeces. The highest counts were obtained in the faecal specimens, whilst those in the transverse colon were 2-3 logarithmic values lower and were lower again in the terminal ileum. Faecal sampling cannot

give information on the composition and localisation of epithelial and cryptal communities (Savage, 1977).

Even in the proximal colon, there are different ecological niches such as the wall of the gut versus lumen and the solid versus the liquid phase, which may differ, in their bacterial populations. The interaction of bacteria in biofilms (see below) should also be considered (Macfarlane *et al.*, 2000).

**Table 1.5**                      **Flora in terminal ileum, caecum and faeces of adult humans**

Species	Mean Log <sub>10</sub> Viable Count/Gram Intestinal Material		
	Terminal Ileum	Caecum	Faeces
Enterobacteria	3.3	6.2	7.4
Enterococci	2.2	3.6	5.6
Lactobacilli	<2	6.4	6.5
Clostridia	<2	3.0	5.4
Bacteroides	5.7	7.8	9.8
Gram positive non-sporing anaerobes (Eubacteria and Bifidobacteria)	5.8	8.4	10.0
Number of samples studied	6	2	100

From Hill and Drasar, 1974

#### **1.2.4.1 Mucosal and mucin associated flora**

It is important to consider that the faecal flora represents only the bacteria in the lumen of the gut and this may differ to the bacteria attached to the mucosa or the mucus layer. Most work of the mucosal associated flora has been carried out in animals because of the relative ease of obtaining samples. Lactobacillus cells have been shown to adhere directly to the epithelium of the large intestine in rats and mice (Savage, 1983). Oxygen intolerant anaerobes have been detected in the colonic epithelium of rats, mice and dogs (Savage, 1977). The flora can physically adhere to the epithelia, this may be a weak attachment in the colon but the slow movement of contents and the sluggish peristalsis enable the bacteria to remain

attached (Savage, 1977). Many microbial species may colonise the layers of mucin on the epithelial surfaces (Savage, 1977; Croucher *et al.*, 1983). Mucin itself may serve as a carbon and energy source for the microorganisms (Perman and Modler, 1982). There is limited evidence that the association of the microflora with mucosal surfaces is beneficial. However, microbial cells in close proximity to epithelial cells compete with the host for nutrients and some bacterial metabolites may be detrimental to enterocyte structure and function (Tannock, 1999).

Very little data exist on these differences between the luminal and mucosal flora in humans (Poxton, 1997). One study of biopsy specimens obtained at surgery showed a large number of gram positive organisms adhering to the mucosal layer (Nelson and Mata, 1970). Nelson and Mata, 1970, investigated normal colonic specimens removed from healthy portions of colon during intestinal resections and from biopsies during anal surgery or proctoscopy.  $10^7$  anaerobic bacteria and  $10^6$  aerobic bacteria per gram of tissue were found attached to the gut wall. The most prevalent organisms were anaerobic and microaerophilic streptococci and Enterobacteriaceae. Croucher *et al.*, 1983, studied colon wall flora obtained from four sudden death victims (traffic accidents and heart failure). They showed that the predominant flora associated with the colon wall was anaerobic, the main isolates being bacteroides and fusobacterium. Other studies have indicated intestinal spirochaetes attached to the mucosa (Lee *et al.*, 1971) although culture of these organisms has not proved possible. The human gastrointestinal tract is lined by a columnar epithelium and does not provide sites for adherence of lactobacilli. Whilst spiral-shaped microbial cells have been shown to be associated with human colonic mucosa, evidence for significant association by other bacteria is inconclusive (Croucher *et al.*, 1983; Tannock, 1997). The predominance of anaerobic bacteria next to the mucosa may seem surprising as the oxygen level should be high as it diffuses across the intestinal cells. However, probably due to the presence of facultative anaerobes, oxygen does not appear to be a controlling factor in the growth of anaerobic bacteria at epithelial surfaces.

The bacteria in the colon exist in many different habitats and metabolic niches that are associated with the mucosa, the mucus layer and particle surfaces in the colonic lumen (Macfarlane *et al.*, 2000). Where there are suitable surfaces, bacteria and other micro-organisms have a tendency to form biofilms, composed of a single species or more likely of many species. Studies suggest that an individual bacterium adheres to a surface and this is followed by non-linear proliferation of the cells leading to the formation of the biofilm. These biofilms have altered metabolism allowing greater resistance to antibiotics and other inhibitory factors (Anwar *et al.*, 1990; Van Loosdrecht, 1990).

### 1.3 COLONIC FERMENTATION

Until the 1980's, the colon was largely regarded as a reservoir where undigested material was concentrated and stored and its only other function was maintaining the balance of water and electrolytes in the body (De Schrijver, 1996). However the bacterial flora has a metabolic capability of comparable size to the human liver. Any carbohydrate escaping digestion and absorption in the small intestine is available for anaerobic metabolism by the colonic flora.

Non-starch polysaccharides, resistant starch (Englyst *et al.*, 1992), oligosaccharides and mucins are all subject to fermentation processes (Table 1.6; Edwards and Rowland, 1992). It has been calculated that the daily amount of carbohydrate needed to sustain the colonic microflora is 60-70g (Wolin, 1981; Cummings *et al.*, 1970). Much less than this enters the colon from undigested dietary carbohydrate. In studies with ileostomy patients this was found to be 25-35g (Schweizer *et al.*, 1990). This difference suggests that host secretions are important substrates for bacterial fermentation in the colon.

It is estimated that of the 70 g needed for bacterial growth, between 8 and 40g/ day may be resistant starch (RS), 8-18g/ day non-starch polysaccharides, 2-10g/ day unabsorbed sugars and 2-8 g/ day oligosaccharides (Macfarlane and Cummings, 1991; Macfarlane *et al.*, 1995). Resistant starch is defined as 'the



**Table 1.6**                      **Possible substrates available for fermentation**

Substrate	Comment
	Soluble fibres are extensively fermented.
Plant cell wall carbohydrates	Insoluble fibres are much more resistant to fermentation
Dietary fibre	
<b>Prebiotics</b>	Fructo-oligosaccharides from inulin
Oligosaccharides	Rafinose, stachyose present in legumes
	Fructans present in onions, garlic.
	Dextrins formed when starchy foods are subjected to heat treatment.
	Galacto-oligosaccharides from human milk
Starches – Resistant starch	RS type 1 physically inaccessible
	RS type 2 resistant starch granules
	RS type 3 retrograded amylose
	(Englyst <i>et al.</i> , 1992)
Unabsorbed Protein	Protein fermentation produces branched chain SCFA,
Unabsorbed Fat	Glycerolipids, steroids and waxes
Polyphenols	Including lignans and isoflavonoids
Inositol phosphate	Phytate is the main source of phosphorous in foods of plant origin
<b>Endogenous sources</b>	
Mucus	From colonic secretion and from the upper gut
Protein	From endogenous enzymes and other gut secretions
Fat	From sloughed off cells.
Urea	Diffused into the gut from the blood and tissues.

sum of starch and products of starch degradation not absorbed in the small intestine of healthy subjects' (Asp, 1992). However, it is now estimated that only 3.5 – 6.0 g/ day RS is ingested in Europe (Dysseler and Hoffem, 1994) so these figures may need to be adjusted. Endogenous carbohydrates such as mucin and chondroitin sulphate may contribute between 2-3g/ day. Other substrates that contribute are proteins and peptides (up to 25g/ day), bacterial secretions, lysis products and sloughed epithelial cells (Macfarlane and Macfarlane, 1995; Macfarlane *et al.*, 1988).

Colonic fermentation is the microbial anaerobic degradation of various substrates, mainly carbohydrates. Carbohydrates are degraded via the Embden-Meyerhof pathway to short chain fatty acids (acetate, propionate, butyrate, lactate) and gases ( $\text{CO}_2$ ,  $\text{H}_2$ ,  $\text{CH}_4$ ). Hydrogen may be further removed by some bacteria or may be excreted either in breath or flatus. The microflora can also dispose of hydrogen by methanogenesis, dissimilatory sulphate reduction or acetogenesis (Gibson *et al.*, 1990). There is some variation in the SCFA produced in the colons of individuals due to the substrates and bacteria available; however, there is an overall trend. The major SCFA is always acetic acid with less propionic and then butyric acid.

Rapid fermentation can also result in intermediates, ethanol, succinate, lactate and pyruvate, which may be further fermented allowing more energy gain from the process. The branched chain SCFA are the products of amino acid fermentation (Macfarlane and Allison, 1986; Macfarlane *et al.*, 1992).

The fermentation process is complex and depends largely on the available substrates and the composition of the microflora. The colonic microflora are themselves influenced by substrates, pH and moisture content. Substrates and end products may influence the metabolism of each other and the gut flora. Metabolic end products of one species are the growth substrate of another species. End products may be excreted in the faeces or absorbed from the colon or utilised by the bacteria. Absorbed products may be utilised by the colonic mucosa, excreted via the breath or re-excreted via the enterohepatic circulation (De Schrijver, 1996).

The factors determining fermentation are not fully understood. The chemistry and tertiary structure of the substrate is likely to be critical (Salvador *et al.*, 1993). However there is very little interpretable data that explains this complex interaction. The presence of bacterial enzymes is important and other environmental factors such as pH, bile acids, mixing rate, may also have some effect. A longer transit time allows more time for slowly fermentable polysaccharides to be fermented (Van Soest *et al.*, 1982). Shorter transit times are

less efficient for fermentation (Gibson, 1996). If a polysaccharide does not form part of the normal diet the enzymes for its breakdown may not be present. When this occurs, the enzymes have to be induced or individual bacteria need to increase in population, and adaptation to a particular polysaccharide may take several weeks (Daly *et al.*, 1993). Walter *et al.*, 1986 showed that the fermentative capacity of rats was still changing after four weeks of ingestion of a new fibre and therefore the results of feeding trials of less than this duration may not give accurate results.

Fermentation is difficult to study in adults for similar reasons to the study of the colonic microflora. There are several different approaches to the study of colonic fermentation and these will be discussed in detail in Chapter 3.

Faecal SCFA may not reflect the true bacterial activity as they are the net result of both production and absorption and may not represent events in the proximal colon. Other data has been derived from *in vitro* fermentation models.

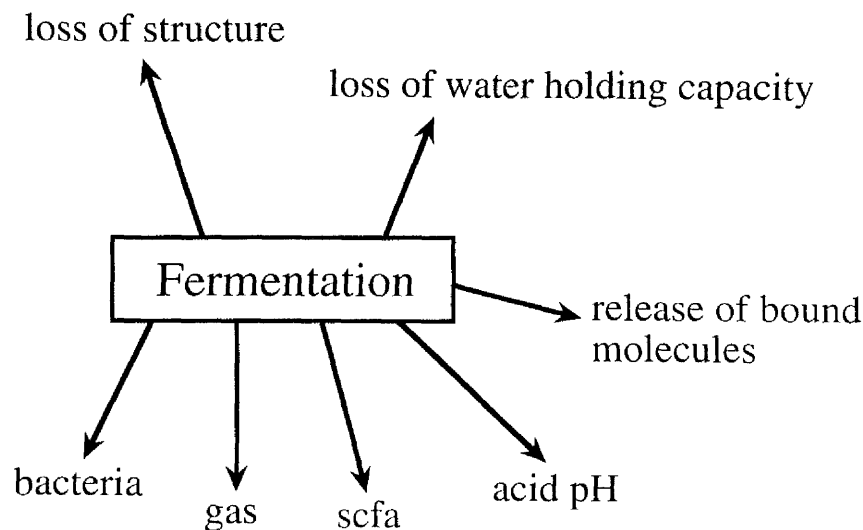
### **1.3.1 Consequences of fermentation**

Fermentation of carbohydrate has many consequences (Figure 1.1). The major effects of carbohydrate fermentation include the destruction of dietary fibre structure with loss of water holding capacity, production of SCFA, production of gases, decreased pH, release of bound molecules, production of bacterial cells and changes in bacterial enzymes.

The water holding capacity of a fibre is a major determinant in its effect on stool output. Carbohydrates that are extensively fermented lose their structure and water holding capacity (McBurney *et al.*, 1985). As a consequence their action on stool output may be prevented or reduced. The carbohydrates that are least fermentable tend to have the greatest effect on stool output, although there are exceptions to this rule. If a carbohydrate is rapidly fermented there is an increase in bacterial mass, which will have its own water holding capacity (Stephen and

Cummings, 1980) and thereby increase stool output. This is accompanied by an increased turnover of nitrogen in the gut. Fermentation increases the requirement of the bacteria for nitrogen by increasing bacterial growth (Cummings, 1984). Increased microbial nitrogen excretion leads increased faecal nitrogen excretion (Stephen and Cummings, 1979). The main source of nitrogen is ammonia from urea or protein. When fermentation does not occur, ammonia is absorbed and passes to the liver for conversion to urea (Cummings, 1984).

Figure 1.1 Consequences of fermentation

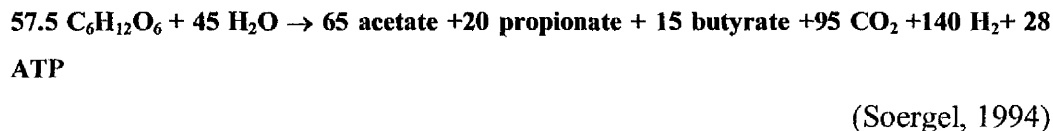
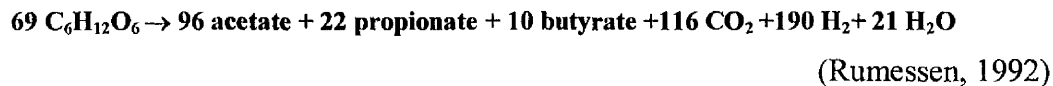
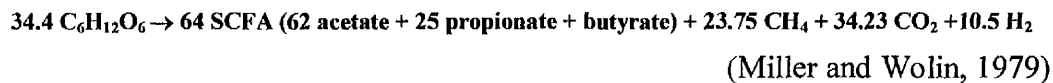


Increases in stool output may have nutritional implications including increased loss of nitrogen, energy, water and electrolytes. There may also be an increased loss of bile acid metabolites and other possible toxins. These losses may decrease the risk of mucosal damage. Fibre fermentation by bacteria may release sequestered molecules such as calcium and bile acids (Eastwood and Hamilton, 1968), which are then available for absorption. Bacteria deconjugate and dehydroxylate bile acids altering the composition and properties of the bile acid pool (Phillips and Devroede, 1979) and also producing possible colonic cancer promoters (Hill and Drasar, 1975). Bile acid metabolites and bacterially

hydroxylated fatty acids may cause colonic secretion and stimulate colonic motility (Ammon and Phillips, 1982).

#### 1.4 PRODUCTION OF SCFA

SCFA produced during fermentation have several important actions, but first it is important to consider the amount produced during fermentation. Miller and Wolin, 1979 derived an equation for overall fermentation in the gut. This equation was based on measured amounts of faecal SCFA and known production rates of intestinal gases. This equation has been updated since then by different researchers:



Due to the difficulties in measuring fermentation *in vivo*, it is unclear which of these equations is closest to real reactions. In healthy adults, 10% of SCFA are excreted in faeces with a molar ratio of approximately 57:22:21 for acetate/propionate/butyrate (Szyliet and Andrieux, 1993). These SCFA ratios were maintained between the age of 23 and 70. A change in diet, from normal diet to one containing only carbohydrates, did not appear to affect this ratio although there was a decrease in overall total SCFA concentration. This suggests that a change in diet has less effect than might be expected. Other studies have shown the opposite, that total SCFA concentrations are unaffected but patterns of SCFA do change (Cummings *et al.*, 1978; Fleming and Rodriguez, 1983). There is strong evidence from *in vitro* studies (Englyst *et al.*, 1987) and *in vivo* animal studies (Berggren *et al.*, 1993) that the substrate entering the colon directly affects the products formed. Starch fermentation has been shown to give high yields of

butyrate whereas pectin fermentation yields high acetate (Englyst *et al.*, 1987; Scheppach *et al.*, 1988). The ingestion of large doses of lactulose (160g/ day) increased the percentage of acetate and lactate produced at the expense of other SCFA (Mortensen and Nordgaard, 1995). However, feeding studies often give a single substrate in high quantities either alone or mixed with the normal diet over a period of time. Eating large amounts of a particular substrate like this does not resemble the mixed carbohydrate sources found in normal diet. Very little branched chain SCFA, produced by protein fermentation, and lactic acid, produced by rapid fermentation, appear in the faeces of adults mainly because they are used by the bacteria for new cell growth.

#### **1.4.1 Actions of SCFA**

The end products of fermentation have many different metabolic fates (Table 1.7) and the SCFA produced have a number of physiological actions.

Acetate, propionate and butyrate are the principle SCFA produced by colonic fermentation (Cummings *et al.*, 1987). SCFA are rapidly absorbed (McNeil *et al.*, 1978) by the colon promoting water and sodium absorption by  $\text{Na}^+ / \text{H}^+$  exchange (Ruppin *et al.*, 1980) and may therefore prevent osmotic diarrhoea. It has been postulated that up to 7% of the energy requirement of normal adults could be provided by the SCFA (Rechkemmer *et al.*, 1988). It is now considered that fermentation of carbohydrate has an average energy value of 2 Kcal/ g (Livesey, 1990). The true value may sometimes be nearer 4 Kcal/ g but the energy gained by utilisation of SCFA must be offset by the increased faecal losses.

SCFA are metabolised by mucosal cells. SCFA not metabolised in the mucosa enter the portal vein after absorption (Dankert *et al.*, 1981). The concentrations of acetate, propionate and butyrate are much higher in portal blood than peripheral venous blood. However, approximately 90% of butyrate and 10-50% of propionate is metabolised in the colonic mucosa (Bergman, 1990). Acetate

escapes complete uptake and metabolism in the gut mucosa and clearance in the liver and there is an increased proportion of acetate in portal blood compared with the gut lumen (Mortensen and Nordgaard, 1995). Eventually acetate accounts for approximately 90% of SCFA in peripheral blood (Cummings, 1987). SCFA concentrations decrease from 60-150mmol/ l to 0.2-0.3 mmol/ l to approximately 0.06mmol/ l in large intestine, portal blood and plasma respectively (Cummings, 1987).

**Table 1.7 Metabolic fate of fermentation end-products**

End Product	Metabolic Fate
Acetate	Mostly energy source for muscle, kidney, heart and brain (Remesy <i>et al.</i> , 1992) Substrate for lipogenesis
Propionate	Metabolised by liver, gluconeogenic, may suppress cholesterol synthesis but not at physiological levels
Butyrate	Main fuel of colonic epithelium, stimulates cell differentiation, apoptosis and cell turnover and health
Ethanol, succinate, pyruvate, lactate	Electron sink products, further fermented to SCFA or absorbed by colon
Hydrogen	Excreted in breath or flatus, metabolised by bacteria to H <sub>2</sub> S, CH <sub>4</sub> or acetate
Methane	Excreted in breath or flatus
Hydrogen sulphide	Absorbed, excreted may damage mucosa
Carbon dioxide	Excreted in breath or flatus
Ammonia	Absorbed, converted to urea in liver, possible co-carcinogen
Branched chain fatty acids	Absorbed and transported to liver, growth promoters of some bacteria
Amines	May serve as reactants in N-nitrosation reactions
Phenolic compounds	Absorbed and metabolised in liver,

Adapted from Gibson, 1996

#### **1.4.1.1 Acetate**

Acetate is usually formed by the oxidative decarboxylation of pyruvate, and butyrate from the reduction of acetoacetate formed from acetate (Cummings, 1981). A variety of actions have been suggested for acetate but its main role is as a fuel source. Acylation (activation for metabolism) of acetate occurs in both the cytosol and mitochondria, whereas activation of propionate and butyrate is solely mitochondrial. Cytosolic acetate is an effective precursor for lipogenesis (Remesy *et al.*, 1992). The uptake of acetate by the liver has been directly correlated to the concentration of acetate in portal blood (Herrmann *et al.*, 1985). Acetate lowers free fatty acid and glucose concentrations in the blood and is used in synthesis of cholesterol (Soergel, 1994).

#### **1.4.1.2 Propionate**

Propionate is a precursor for gluconeogenesis because it is readily taken up and activated in the liver (Rémésy *et al.*, 1992). Propionate has also been suggested as possible regulator of cholesterol synthesis. Dietary propionate has been shown to lower serum cholesterol in rats (Illman *et al.*, 1988) and pigs (Thacker and Bowland, 1981). The mechanism by which this occurs is unclear, possibly propionate inhibits hepatic cholesterol synthesis or redistributes cholesterol from plasma to liver. Wright *et al.*, 1990 have shown that low propionate concentrations (1mmol/ l) had an inhibiting effect on cholesterol synthesis in isolated hepatocytes. This finding supported work carried out by Chen and Anderson, 1986. Other investigators (Illman *et al.*, 1988) have shown that during propionate feeding of pigs the concentration of propionate in portal blood was always less than 1mmol/ l and they saw no effect on hepatic lipid synthesis. The same investigators suggested serum cholesterol was reduced by redistribution of cholesterol from plasma to liver in rats. Fewer studies have investigated propionate metabolism in humans. Venter *et al.*, 1990, fed a group of healthy subjects their normal diet supplemented with propionate for a period of 7 weeks. When compared against a control group, there were no differences in glucose



tolerance tests, inulin response or total cholesterol but there was an increase in high-density lipoprotein cholesterol. Wolever *et al.*, 1991, suggested that propionate may interact with acetate, when a mixture of acetate and propionate was rectally infused there was a partial block of the rise in serum cholesterol seen when acetate alone was infused. The effects of dietary administered propionate may be different from those effects caused by the production and absorption of propionate in the colon (Björck *et al.*, 1996). Rectal infusion of propionate caused a lowering of total liver cholesterol pools to the same extent as dietary cholesterol but at a much lower dose. This suggests that propionate produced by colonic fermentation may moderate cholesterol metabolism beneficially.

#### **1.4.1.3 Butyrate**

Butyrate is taken up by the colonic wall and does not reach the liver. However, it may have an indirect effect as it is converted to ketone bodies which, may be released into the circulation. Butyrate is a source of acetyl-CoA but its use is not affected by other fatty acids. Human portal blood has low levels of butyrate despite there being active butyrate production in the colonic lumen. The low levels are probably the result of active butyrate metabolism in the colonic epithelia.

The importance of butyrate as a preferred substrate for colonic epithelial metabolism is now well established (Roediger, 1982). Sakata, 1983, suggested it may stimulate the proliferation of the normal epithelium. Kruh, 1982, observed various properties of butyrate including arrest of cell growth, alteration of gene expression, modification of cell morphology and suppression of cancer-specific properties of transformed cells. All these attributes were shown to be reversible when butyrate was removed. Butyrate has antineoplastic effects on colonic, ovarian and pancreatic carcinoma cell lines in vitro (Soergel, 1994).

Butyrate is the preferred energy source for the colonic mucosa where it is metabolised to CO<sub>2</sub> and ketone bodies. Butyrate may account for 80% of oxygen

consumed by colonocytes in the human (Roediger, 1980). Ulcerative colitis has been associated with a lack of luminal SCFA and impaired mucosal metabolism of butyrate (Scheppach *et al.*, 1992). Improvements in the disease have been shown after treatment with butyrate enemas. Butyrate has also been thought to play a possible role in colon carcinogenesis because of its effect on cell differentiation (Mortensen and Nordgaard, 1995) and apoptosis (Hague *et al.*, 1993; Hague and Paraskeva, 1995). Various studies have investigated faecal SCFA levels in colonic neoplasia and results are inconclusive. A lower ratio of butyrate and higher ratio of acetate was shown in colon polyp and cancer patients (Weaver *et al.*, 1988a). Lower levels of faecal butyrate and propionate (but not statistically significant) were shown in patients with rectal cancers compared with those with more proximal cancers (Vernia *et al.*, 1989). No differences were seen in a study comparing healthy subjects with patients with previous colonic adenomas or colonic cancer (Clausen *et al.*, 1991).

Rapid fermentation in the colon usually results in lactic acid production but also lowers the pH. This pH may have inhibitive effects on various bacterial pathways. Bile acids may be precipitated, reducing their absorption and the 7- $\alpha$ -dehydroxylase enzyme, involved in bacterial bile acid metabolism, will be inhibited. Bacterial production and absorption of ammonia may be reduced (Swales *et al.*, 1970; Vince *et al.*, 1978) and colonic proliferation may be stimulated (Lupton *et al.*, 1988).

### **1.5 OTHER BACTERIAL ACTIVITIES**

Increased intake of fermentable carbohydrates may increase the activity of  $\beta$ -glucuronidase,  $\beta$ -glucosidase and azoreductase, which are involved in the metabolism of exogenous molecules such as drugs and possible carcinogens (Rowland, 1988).

There has been more recent evidence that fermentation products may be beneficial or detrimental to health. It has been suggested that large bowel diseases may be

directly related to inadequate intake of dietary fibre (Burkitt, 1973; Trowell, 1978). Fermentation end products have been related to lower serum cholesterol in rats (Illman *et al.*, 1988; Thacker and Bowland, 1981) and lower blood glucose levels in humans (Wolever *et al.*, 1991). Butyrate has been implicated in the prevention of inflammatory diseases (Scheppach *et al.*, 1992; Vernia *et al.*, 1988) and cancer of the colon (Roediger, 1996). Consequently colonic fermentation has been the subject of much investigation (Soergel, 1994; Hill, 1995; Nordgaard and Mortensen, 1995).

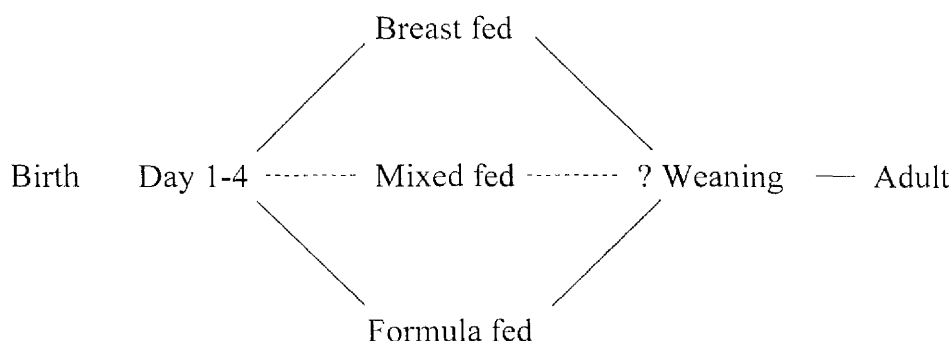
Carbohydrate fermentation and its consequences has been well investigated in the adult but knowledge of the colonic microflora of neonates and how fermentation develops in infancy, during weaning and through early childhood, is limited.

## 1.6 CRITICAL STAGES IN COLONISATION OF THE INFANT INTESTINE

The bacterial colonisation of the neonatal gut may be critical, not only in determining the health of the infant but also establishing the colonic flora of the adult and thereby having an effect on health in later life.

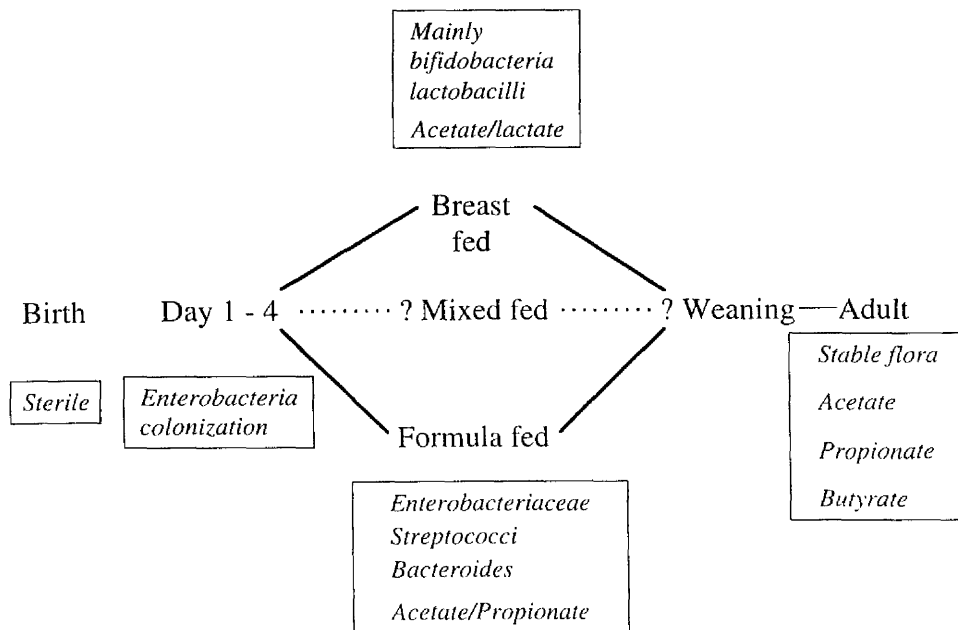
There are two critical stages of bacterial colonisation in infancy; birth and weaning (Figure 1.2).

Figure 1.2 Critical stages in the development of the colonic microflora



The interaction between the colonic microflora and nutrition begins at birth. The gut of the new-born infant is sterile but is quickly colonised after contact with several environmental factors including the bacteria on mother's skin and vagina (Nolte, 1977; Zetterstrom, 1994). The colonisation develops through the first few weeks of life and is dependent on feeding practice (Figure 1.3). At the start of weaning, the infant is introduced to a variety of new substrates, in particular dietary fibre and starch, which may have a major impact on the gut flora. It is therefore important to consider changes to the infants diet during the first year of life.

Figure 1.3 Knowledge of the colonic microflora at critical stages of development



## 1.6.1 Infant Feeding

### 1.6.1.1 Dietary Recommendations

The COMA Report 'Weaning and the Weaning Diet' (Department of Health, 1994) states that "*breast milk provides the best nourishment during the early*

*months of life. Mothers should be encouraged and supported in breastfeeding for at least four months and may choose to continue to breastfeed as the weaning diet becomes increasingly varied”.*

Human milk is considered to be the ideal food for the infant, at least until 5-6 months old, ensuring optimal growth and development (ESPGAN, 1982). One unique property of human milk is that it varies from mother to mother, from day to day during the day and even during a feed (Hytten, 1954). The importance of these differences are not fully understood (Forsyth, 1992). Formula milks have been developed over the years that try to mimic breast milk as closely as possible in their composition but cannot imitate these variations. Research continues in order to develop a formula feed which may not be exactly the same composition as human milk but does achieve the physiological effects seen in breast-fed infants.

### 1.6.2 Differences in breast and formula milk composition

As well as the composition of breast and formula milk, follow-on milks and cow's milk also need to be considered. Table 1.8 gives the composition of all the different types of milk.

**Table 1.8 Difference in the major components between breast, formula and cow's milk**

	Breast milk	Infant formulas	Follow-on milks	Whole cow's milk
Energy kJ	290	285 -290	270 -285	285
(kcal)	(70)	(67 - 70)	(65 - 67)	(67)
Protein (g)	1.3	1.5 - 1.9	2.0 - 2.9	3.4
Vitamin D (µg)	0.01	1.0	1.1 - 1.2	0.02
Iron (mg)	0.08	0.4 - 0.7	0.7 - 1.2	0.05
Saturated fat (g)	2.1	1.0 - 1.9	1.2	2.5
Sodium (mmol)	0.6	0.6 - 1.1	1.3 - 1.5	2.2

Figures given per 100g of feed (made up with water according to manufacturers instructions where necessary)

Adapted from Wharton, 1990

### 1.6.2.1 Carbohydrates

The main carbohydrate in human milk is lactose and constitutes about 80% of the total carbohydrate in human milk although there are small amounts of glucose, galactose and oligosaccharides present. Oligosaccharides are complex carbohydrates that are composed of D-glucose, D-galactose, L-fucose, N-acetylglucosamine and N-acetylneuraminic acid (sialic acid). They may be acidic or neutral depending on the presence of sialic acid (Kunz and Rudloff, 1993). Oligosaccharides are synthesised in the mammary gland by the action of several enzymes, which add specific monosaccharides to the core structure. Human milk contains much more lactose than cows' milk (7g and 4.8g/ 100ml respectively; Hambraeus *et al.*, 1977) and provides about 39% of the energy content compared to 27% in cows' milk. It is thought that lactose may be specific to infancy. In all mammals, except man, the enzyme lactase is not found after infancy. Although it persists into adulthood, particularly in Europeans, many populations do not tolerate lactose very well after middle childhood (Akre, 1989). Lactose facilitates calcium and iron absorption and appears to encourage *Lactobacillus bifidus* to colonise the gut. This colonisation is aided by the presence of a nitrogen containing carbohydrate, the bifidus factor. The growth and metabolism of lactic acid bacteria lower the pH of the gut and so discourage the growth of pathogenic bacteria.

Oligosaccharides are also higher in human milk, 1-1.2g/ 100ml in mature milk, compared with cow's milk, which only contains 0.1g/ 100ml. The amount of oligosaccharides in colostrum is even higher, 2-2.5g/ 100ml. Oligosaccharides represent about 27% of the total carbohydrate in the colostrum, decreasing to 19% at day 30 and by day 60 they have reached about 15-16% (Coppa *et al.*, 1993). Acetyl-lactosamine is present in these sugars which promotes the growth of *Lactobacillus bifidus* (Bezkorovainy *et al.*, 1979). Studies by Petschow and Talbott, 1991 showed that it did not appear to promote the growth of *B.infantis*, *B. brevis* or *B.longum*.

The oligosaccharide portion of glycolipids and glycoproteins are used by enteropathic organisms for targets for attachment of whole bacteria and toxins. By acting as a receptor competing with intestinal ligands the oligosaccharides of human milk may prevent this. Neutral oligosaccharides from colostrum were shown to cause inhibition of adhesion to uroepithelial cells of *E. coli* isolated from an infant with urinary tract infection (Coppa *et al.*, 1990). Evans *et al.*, 1988, have shown that sialyl ( $\alpha$ 2-3) lactose inhibited haemagglutination of *Campylobacter pylori* and of S-fimbriae carrying strains of *E. coli* (which may cause meningitis and neonatal sepsis in new-borns). However, Schroten *et al.* (1993) concluded that its concentrations in human milk might be too low to exert a significant effect.

Another important function of human milk oligosaccharides is to provide the infant with sialic acid. Man has the ability to synthesise sialic acid from simple sugars and phosphoenolpyruvate (Carlson, 1985) but its synthesis in the neonate has not been studied. It is reported to cause early and long-term modifications of behaviour (Morgan and Winick, 1980). Human milk contains about 1g/ l sialic acid during the first week of lactation which then decreases to 250mg/ l at 6-8 weeks (Carlson, 1985). In contrast, only 50-70 and 10-30 mg/ l of oligosaccharide derived sialic acid is found in whey and casein predominant formulas respectively (Carlson, 1985).

Carbohydrate is an important factor in the bacterial colonisation of the colon and as such it will be discussed in more detail in Chapter 7.

#### **1.6.2.2 Protein**

Human breast milk contains less protein than the milk of any other mammal (Akre, 1989). The protein content of human milk falls rapidly over the first days of lactation. Colostrum has approximately 2% protein, transitional milk about 1.5% and mature milk about 1%. The major proteins found in human milk are casein and whey proteins. Caseins are phosphorus-containing proteins that occur

only in milk. The whey proteins, such as lactalbumin, lactoferrin, immunoglobulins and lysozyme, are synthesised in the mammary gland. The whey:casein ratio falls from 90:10 in early lactation to 55:45 in mature milk (Kunz and Lönnerdal, 1992). There are two types of infant formula available in the UK, whey-dominant or casein-dominant. The casein-dominant type is based on the entire cows' milk protein and has a casein:whey ratio of 80:20. The whey-dominant type is based on modified cows milk protein and has a casein:whey ratio of 40:60 which is more like breast-milk (Balmer and Wharton, 1989). Whey-dominant formulas are recommended from birth for infants that are not to be breast-fed at all (Department of Health, 1994). Human whey proteins consist mainly of  $\alpha$ -lactoglobulin whereas the dominant bovine whey protein is bovine  $\beta$ -lactoglobulin, which has no human equivalent (Inch, 1994). The other main whey proteins in human milk, lactoferrin, immunoglobulins and lysozyme have protective roles. Lactoferrin withholds iron from invading organisms by its high affinity for iron as well as its slow rate of change to a conformation in which the iron site is exposed (Chung and Raymond, 1993). A synergic mechanism with lysozyme and IgG has been suggested for lactoferrins antibacterial action (Iyer and Lönnerdal, 1993). Secretory IgA antibodies are active against a range of bacterial, viral, fungal, parasitic and food antigens. The secretion of lysozyme increases during lactation, it has a role in catalysis of breakdown of specific molecular bonds in bacterial cell walls. These three components may act synergistically to enhance the infant's defences against infection.

The main casein protein found in human milk is  $\beta$ -casein whereas cows milk have predominantly  $\alpha$ -caseins (Kunz and Lönnerdal, 1990). The differences in casein proteins can affect curd formation, which influences gastric emptying and intestinal transit time (Billeaud *et al.*, 1990). Theoretically, whey predominant formulas may be advantageous to new-borns as they form finer, softer curds than casein predominant formulas which leads to faster gastric emptying rates which are more like that of a breast fed infant (Billeaud *et al.*, 1990). Human milk with low (mainly  $\beta$ -) casein content forms small, soft, flocculent curds that are easily digested giving a continuous supply of nutrients. Infant formulas have higher



casein content (mainly  $\alpha$ ) form larger, tougher curds which stay in the stomach of the infant longer, and therefore require higher amounts of energy to enable complete digestion (Daniels, 1987; Riordan, 1993). This may be a problem with reconstituted formula. If it is inadequately diluted, the curd complex of insoluble calcium caseinate, calcium phosphate and fat may be so dense that it causes intestinal obstruction (Jelliffe and Jelliffe, 1978). Human milk contains significant quantities of non-protein nitrogen (25% compared to 5% in cows milk). This is derived from free amino-acids, small peptides, amino sugars, creatine, creatinine and glycolipids (Hambræus *et al.*, 1978). The role of some of these substances is not fully understood. The level of different amino acids in human milk varies. It is low in those known to have a detrimental effect such as methionine (may adversely effect the central nervous system) (Worthington-Roberts and Williams, 1989) and high in others that the infant cannot synthesise well such as taurine, which may have a role in brain maturation (Sturman *et al.*, 1977).

#### **1.6.2.3 Fat**

Fats are vital for normal growth and development. They provide energy, supply essential fatty acids and are the vehicle for fat- soluble vitamins and hormones (Goedhart and Bindels, 1994). Approximately 50% of the energy in human milk is due to the fat (Giovanni and Agostoni, 1991), and this fat is more easily digested than that in cows' milk.

The total fat content of human milk can vary from mother to mother and this is directly related to the maternal diet (British Nutrition Foundation, 1992). Generally the fat content of the first milk is low and the hindmilk shows a threefold increase in fat. The majority of the fat content is present in the form of triglycerides but there are also small amounts of phospholipids, cholesterol, diglycerides, monoglycerides, glycolipids, sterol esters and free fatty acids.

Human milk contains a larger amount of cholesterol than cows' milk or commercial infant formula. There have been two suggested roles for this higher cholesterol level. It is important in the myelin synthesis of the rapidly growing central nervous system and its presence in breast-milk stimulates the enzymes necessary in later life for cholesterol degradation (Jelliffe, 1975; Joote *et al.*, 1991).

The triglycerides of human milk contain more than 150 different fatty acids. The fatty acid composition of human milk is relatively stable, 46% saturated and 54% unsaturated fatty acids (Jensen, 1989). Many of these unsaturated fatty acids take the form of long chain polyunsaturated fatty acids, which are particularly important for brain growth and myelination. Two of the polyunsaturated fatty acids from which the longer chain fatty acids are derived are linoleic and  $\alpha$ -linolenic acid. These are not synthesised by the infant or mother but the mother obtains them from plant sources in the diet (Sinclair, 1992). Recent research has shown that human milk contains docosahexanoic acid (DHA) and arachadonic acid (AA), the long chain derivatives of which  $\alpha$ -linolenic and linoleic acid are the respective precursors.

Until recently neither DHA or AA were added to infant formulas. Formula fed infants, especially those born prematurely, may not be able to convert the precursors to the active long chain fatty acids. If DHA is not supplied, other fatty acids, including saturated fats, are substituted and this potentially can lead to different concentrations of polyunsaturated fatty acids in the brain and retinal tissues (Farquharson *et al.*, 1992). Consequently, the ESPGAN Committee on Nutrition (1991) and the British Nutrition Foundation (1992) recommend that formula feeds for premature infants are enriched with DHA and AA. The British Nutrition Foundation (1992) also recommend the addition of AA and DHA to infant formulas for term infants in amounts similar to human milk.

#### 1.6.2.4 Minerals and Trace Elements

The major minerals in breast milk are iron, calcium and phosphorous. Mineral concentrations are lower in human milk than both cows' milk or infant formulas. Despite their low concentration, they are adequate for the infants' needs because of their high bioavailability and they are also suitable for the infants' metabolic capabilities. The high mineral and protein content of formulas will affect the amount of mineral and protein waste products going through to the kidney and this could have adverse effects due to the immaturity of the kidney in early life.

Calcium and phosphorus levels are significantly lower in human milk than in infant formulas (Anderson, 1992). It has been found that there is six times more phosphorus and four times more calcium in cow's milk than in breast milk. Calcium is more efficiently absorbed from human milk and therefore bone mineralisation is similar in breast-fed and formula-fed infants (Mimouni *et al.*, 1993). Even at levels of calcium as low as 200-300mg/ day there is adequate bone mineralisation. The absorption of calcium in breast-fed infants is due to a variety of factors. These include the high calcium:phosphorus ratio, high lactose content, low buffering capacity and the composition and structure of the fat.

Human milk has a very low iron content of about 0.5mg/ l but breast fed infants rarely deplete these stores before four months of age (Siimes *et al.*, 1979; Calvo *et al.*, 1992). This is because of the highly efficient absorption of iron from breast milk, 50-70% being absorbed. The iron in infant formulas is in the form of an inorganic salt and only about 10% is absorbed (Akre, 1989; Williams, 1993).

The effects of calcium and iron may have a major impact on the colonic flora and will be discussed later in the chapter.

Zinc is also present in breast milk in small amount but high availability. It is utilised by the infant without affecting copper or iron absorption (Akre, 1989). Other trace elements, copper, cobalt and selenium are present in higher levels in human milk than in cows' milk.

### **1.6.2.5 Vitamins**

All vitamins required by the infant are found in breast milk, however amounts can vary from mother to mother. The maternal diet and mothers vitamin status has a strong influence on the vitamin content of the breast milk. The vitamin levels in infant formulas are based on values found in mature breast milk.

## **1.7 BENEFITS OF BREAST-FEEDING**

Over the past twenty years there has been increased encouragement to new mothers to breast-feed. This included the launch of the government's joint breast-feeding initiative and publication of a booklet on breast-feeding by the Royal College of Midwives (DoH, 1988; Royal College of Midwives, 1988). Several studies have been carried out to provide evidence that 'breast is best' for the newborn. There is evidence of benefits of breast-feeding immunologically, nutritionally and psychologically. Breast-feeding is also the cheapest means of feeding a child in the first six months of life, even though there is an energy cost to the breast-feeding mother.

Breast-feeding promotes a good relationship between mother and child (Jelliffe and Jelliffe, 1971). Previous work has shown that intimate mother-child contact shortly after delivery leads to maternal bonding and favours breast-feeding (Sosa *et al.*, 1976). Early mother-child contact has long range effects in the first year of life. It is difficult to determine which is the most important factor, desire to breast-feed, skin to skin contact, the suckling stimulus, breast-feeding itself or maternal contact.

Human milk provides sufficient energy, macro and micronutrients for rapid growth and development in the first four months of life. Heinig *et al.*, 1992, showed that infants fed solely formula feeds weighed more between 7 and 18 months and 6 and 18 months (male and female respectively) than breast fed infants. The data suggested that breast-fed infants gain weight more slowly in the

first year of life particularly in the last 9 months. These findings were confirmed by Savage, (1998), but only in females and not in males. The difficulty is knowing whether growth is delayed in breast-fed infants or accelerated in formula-fed infants, and ultimately which is best.

Several studies have linked breast-feeding with less gastrointestinal infections in developing countries (Plank and Milanesi, 1973; Habicht *et al.*, 1986). At least some of this protective effect was because of contaminated water used in making up formula feeds. There has been much less agreement about the protective effect of breast-feeding on gastrointestinal effects in developed countries. Bauchner *et al.*, 1986 reviewed all investigations in this area since 1986. These authors concluded that most of the studies identified were inadequate and had methodological flaws, however, their conclusions also suggested that the protective effect of breast-feeding in developed countries was minimal.

Howie *et al.*, 1990, undertook a study to assess the relationship between breast feeding and infant illness in the first two years of life. Infants that were breast-fed for the first thirteen weeks of life had reduced rates of gastrointestinal disease during this period and the benefit persisted for up to one year of age. These benefits remained after all confounding variables had been taken into account. During the first thirteen weeks of life, breast-fed infants had one-third the amount of gastrointestinal disease of formula-fed infants. This investigation also showed a significant effect of breast-feeding (for more than thirteen weeks) in protecting against respiratory illness. However, there was no consistent protective effect against ear, eye, mouth or skin infections, infantile colic, eczema or nappy rash.

Infants in the study of Howie were reinvestigated at the age of seven years (Wilson *et al.*, 1998). This follow-up study showed that the probability of ever having respiratory illness, in children who had received breast milk for at least 15 weeks, was consistently lower. There was no significant difference between formula-fed infants and those who had received partial breast-feeding. Children who had been formula-fed as infants had significantly higher systolic blood

pressure than those who had received breast milk. The study concludes that exclusive breast feeding significantly reduces the chance of having respiratory illness in childhood and may have beneficial effects on childhood health and subsequent adult disease.

Other work has shown an effect against otitis media (Duncan *et al.*, 1993). This study showed that breast-feeding reduced the risk of otitis media by about 50% when compared with infants that did not breast-feed or did not exclusively breast-feed. It was postulated that the effect could be mechanical because of the way infants are held during feeding or that the levels of secretory IgA found in breast milk are protective.

The newborn infant is more susceptible to infections than older children or adults because barrier function, innate immunity and specific immunity are immature at birth (Wold and Hanson, 1994). The barriers of skin and mucosal membranes are more permeable than adults because they are thinner and also do not have a protective covering of microflora. The innate immune system is still quite immature at birth. Phagocyte levels in the blood are almost as high as adults but the total pool of phagocytes that can be mobilised on infection is small. Complement components are often low in new-borns. In contrast, the specific immune system is well-developed at birth except for the antibody response of the IgG2 and IgG4 subclasses (Wold and Hanson, 1994). The new-born infant has the capacity to react with specific immunity to almost all antigens but a primary response has to take place for each new antigen that is encountered. In comparison to a secondary response, this takes a longer time to develop, is lower in magnitude and has a different isotype pattern. Because of this, the new-born infant is at risk of developing overwhelming infection before the specific immune response is activated.

The infant has ready-made defence factors from its mother, first by the passage of IgG across the placenta and then through breast milk. Secretory IgA comprises over 90% of the immunoglobulins in human milk (Butte *et al.*, 1984). Human

breast milk contains 5-10 g/ l secretory IgA in early colostrum and 0.3-1 g/ l in mature breast milk and high levels even one year after delivery. The new-born infant cannot produce significant amounts of SIgA and relies on breast milk for this essential antibody. The other immunoglobulins IgM and IgG are found in much lower quantities in the milk, 0.06 and 0.1 g/ l in colostrum and 0.06 and 0.01 g/ l in mature milk respectively (Wold and Hanson, 1994).

Secretory IgA is formed by the combination of dimeric IgA with the secretory component exposed on the basolateral aspect of the mammary gland epithelial cells (Hanson and Brandtzaeg, 1989). The whole complex is internalised and released to the milk as secretory IgA. The secretory component-mediated transport system exceeds that of the IgA production so the milk contains free secretory component as well as secretory IgA. Sensitised plasma cells are transported from the gastro-intestinal and broncho-tracheal associated lymphatic tissues to multiple mucosal surfaces including breast alveoli during lactation. The “homing” of these cells to the breast seems to be activated by lactogenic hormones. The antibodies in human milk are directed against a wide range of bacterial and viral antigens and also against food proteins in the mother’s diet.

Milk immunoglobulins may interact with microorganisms by other mechanisms. Secretory IgA has N-linked oligosaccharide chains that act as receptors for type-1 fibriated *E. coli*. This then reduces their attachment to colonic epithelial cells (Wold *et al.*, 1990). There are two subclasses of IgA, IgA1 and IgA2, which are found in equal quantities in human milk. IgA1 also has O-linked oligosaccharide chains which recognise *Pseudomonas aeruginosa*. The oligosaccharide chains of the secretory component inhibit *Helicobacter pylori* adhering to the gastric epithelium (Boren *et al.*, 1994). Studies in Mexico (Ruiz-Palacios, 1990) showed that breast-feeding protected against diarrhoea. Children in this study who were breast-fed and developed diarrhoea did not have secretory IgA antibodies to the glycine acid-extractable common antigen of *Campylobacter*. Table 1.9 shows the anti-infectious factors in breast milk.

### 1.7.1 Breast-feeding practice in the UK

Breast-feeding practice in the UK has changed in the last 50 years several times. The introduction of National Dried Milk in the mid 1940s greatly contributed to the decline in breast-feeding that took place in the mid 1900s. There was then a steady decline until about 1970. In the late 1960s, a paper was published linking National Dried Milk with hypertonic dehydration (Simpson and O'Duffy, 1967) and this precipitated an increase in breast-feeding. After 1970, there was a steady growth in breast-feeding, but only until 1980, since when, there has been no subsequent increase. Also since 1980 there has been no change in the length of time that mothers breast-feed for, most giving up within the first few weeks. In Scotland, only half of all mothers breast-feed at birth and there is a rapid decline with age (OPCS, 1995; Table 1.10). This is lower than the number of mothers who begin breast-feeding in England and Wales. It must also be remembered that there are wide variations between areas, ranging from 21.1% in Motherwell, Lanarkshire to 59.1% in Lerwick, Shetland (Ferguson *et al.*, 1994). Nationally it has been shown that mothers of higher social class, mothers who had left full-time education after the age of 19, older women, women in the south of Great Britain and non-smokers were more likely to breast-feed (Savage, 1998). These criteria, as well as those mothers who had previously breast-fed, were also true of those who continued to breast-feed longest (OPCS, 1995).

**Table 1.9 Anti-infectious factors in human milk**

<b>Factor</b>	<b>Function</b>
"Anti-Staphylococcus factor"	Inhibits systemic staphylococcal infection
Secretory IgA (s-IgA), IgM and IgG	Act against bacteria invading or colonising mucosa
Lactoferrin	Binds iron and inhibits bacterial multiplication
Lactoperoxidase	Kills streptococci and enteric bacteria
Complement (C3, C4)	Promotes opsonisation
Lysozyme	Lyses bacteria
"Ribonuclease-like factor"	Has antiviral activity
"Bifidus factor"	Stimulates growth of bifidobacteria
Lymphocyte	Synthesises s-IgA
Macrophage	Synthesises complement, lactoferrin, lysozyme and other factors

From Mata, 1978.



**Table 1.10 Percentage of breast-feeding at ages up to nine months of age by country (1980, 1985, 1990,1995)**

	England and Wales				Scotland			
	1980	1985	1990	1995	1980	1985	1990	1995
Birth	67	65	64	68	50	48	50	55
1 week	58	56	54	58	44	41	41	46
2 weeks	54	53	51	54	41	38	39	44
6 weeks	42	40	39	44	32	29	30	36
4 months	27	26	25	28	21	22	20	24
6 months	23	21	21	22	18	18	16	19
9 months	12	11	12	14	9	9	9	13

OPCS, 1995. Infant Feeding

Since 1974, there has been an official recommendation that mothers breast-feed their infants. It has been suggested that mothers breast-feed preferably for four to six months, but at least for the first few weeks of life. The 1990 OPCS figures show that mothers in Great Britain are not achieving this aim. However, the decline in breast-feeding from 1980-1990 has been reversed and more mothers are both beginning breast-feeding or are still solely breast-feeding at each age up to 9 months (OPCS, 1995).

Since the campaign to increase breast-feeding, many mothers begin and find that they are, for different reasons, unable to continue. Reasons include having a caesarean or low birth weight infant, delay in putting the infant to the breast (even for an hour), hospital feeding regimes, physical problems with breast-feeding and influences of friends and mother. In some cases, this leads to a complete change to formula milk whereas in others they may continue to breast-feed but supplement with formula feeds. In the study, described in this thesis, all these cases are referred to as mixed feeding. At each age there has been an increase in the percentage of mothers giving formula milk from 1980-1995 (Table 1.11; OPCS, 1995).

**Table 1.11**

**Bottles given to breast fed infants at about six weeks and four months (1980, 1985, 1990 and 1995, Great Britain)**

	Age of infant							
	6-10 weeks				4-5 months			
	1980	1985	1990	1995	1980	1985	1990	1995
	%	%	%	%	%	%	%	%
No bottles given	72	66	61	54	81	76	73	57
Bottles given	28	34	39	46	19	24	27	43

OPCS, 1995, Infant feeding.

### 1.8 WEANING (OR COMPLIMENTARY FEEDING)

The second major critical period in the bacterial colonisation of the gut occurs over the long period during which the child is weaned from milk alone to an adult mixed diet. As the infant begins weaning, they experience many new dietary components, many of which will affect the gut microflora. In addition, the development of the functions of the pancreas and small intestinal mucosa may influence the amount of food that escapes digestion and enters the large intestine (see below). The infant normally receives small amounts of a range of new carbohydrates, many of which are new substrates for the flora, which then begins to change.

Weaning has many definitions but for the purpose of this thesis it is defined as the introduction of any semi-solid or solid food (not liquids, other than water) to the diet of the breast-fed or formula-fed child. It is at this stage of an infant's development that the diet changes from a single food, either breast or formula, to a wide variety of foods. This then enables the infant to satisfy their changing nutritional needs at a period of rapid growth.

The weaning process is vital for infants, not only in the nutritional sense, but also to establish normal feeding habits (Lambert and Hall, 1995). Two questions frequently arise with regard to weaning, 'when to begin weaning?' and 'what foods to give?'

The COMA 1994 report 'Weaning and the Weaning Diet' (Department of Health, 1994) recommends that infants should not receive solid foods before the age of four months and that a mixed diet should be offered by the age of six months.

It is thought that at four to six months the infant is sufficiently developed in terms of renal and gastrointestinal physiology to cope with the introduction of solid foods. This is also a period of rapid growth and development when there is an increasing need for nutrients and energy. The need for iron is a major factor. There is very little iron in breast milk but by 4 months the infant iron stores begin to run out. The actual age of weaning is much less than the recommended 4-6 months, although in 1995 a smaller proportion of mothers (55% compared with 68%) had introduced solid foods by the age of three months than in previous years (OPCS, 1995). Mothers who do not breast-feed are more likely to introduce solids at an early stage (OPCS, 1995). The weaning age of infants who took part in this study is described fully in chapter 4 and but the mean weaning ages were 16.6, 15.7 and 18.5 weeks for breast-fed, formula-fed and infants who had a mixture of breast and formula respectively.

## **1.9 DEVELOPMENT OF DIGESTION AND ABSORPTION**

One major factor in the colonisation of the infant gut is the amount of carbohydrate and other substrates available for fermentation. These are nutrients, which have escaped digestion and absorption in the small intestine. Therefore the exact amount of colonic 'fuel' depends on the capability of the pancreas and small intestine. The development of the GI tract is slow and not complete at birth.

### **1.9.1 Digestion in the Adult**

The digestion and absorption processes in the adult are well established and are summarised in Table 1.12.

**Table 1.12 Summary of digestion and absorption in the adult**

Intestinal site	Carbohydrate	Protein	Fat
Mouth	Chewing breaks up food. Salivary amylase breaks $\alpha$ 1-4 glucosidic bonds in starch. Releases maltose, maltotriose, $\alpha$ limit dextrins	Chewing breaks up food	Chewing breaks up food
Stomach	Amylase inactivated. Gastric disruption and sieving of food through 2mm wide pyloric sphincter. Controlled gastric emptying (half time of about 1 hour).	(As for carbohydrate) Acid and pepsin in stomach releases peptides	(As for carbohydrate) non- bile salt dependent lipase releases fatty acids. Emulsification of fats begins.
Small intestine	Pancreatic amylase breaks $\alpha$ 1-4 glucosidic bonds in starch.	Pancreatic enzymes, trypsin, chymotrypsin, carboxypeptidase and esterase digest proteins and release peptides and amino acids.	Fats emulsified with bile acids, phospholipids etc. Pancreatic lipase, co lipase and bile acids allow fat digestion and release of fatty acids and 2 mono-glycerides.
	Releases maltose, maltotriose, $\alpha$ limit dextrins		
	Brush border enzymes including $\alpha$ dextrinase break $\alpha$ 1-6 glucosidic bonds and $\alpha$ 1- 4 bonds and release glucose that is absorbed by active transport.	Trypsinogen is activated by brush border enterokinase and then activates other peptidases.	Micelles formed with bile acids at critical micellar
	Sucrase digests sucrose and lactase releases galactose and glucose from lactose.	At the brush border or within the cell depending on peptide structure, aminopeptidase releases amino acids.	concentration which move products of fat digestion to mucosal wall where they are released and absorbed by diffusion or specific carriers.
Large intestine	Galactose shares glucose sodium dependent active co-transporter, fructose absorbed by facilitated transport.	Five sodium dependent active transporters for specific amino acids allow absorption into cell.	Fats reassembled in cells and put in chylomicrons released in to lymphatic system. Bile acids reabsorbed in ileum.
	Dietary fibre, oligosaccharides, any starch which escapes digestion and absorption (Resistant starch) fermented by colonic bacteria to short chain fatty acids which are then absorbed. Gases CO <sub>2</sub> and H <sub>2</sub> produced which may then be converted to CH <sub>4</sub> .	Bacterial metabolism of unabsorbed protein or peptides releases branched chain fatty acids and H <sub>2</sub> S. Other more toxic compounds may be released	Unabsorbed fat or bile acid subjected to bacterial metabolism.
	Unabsorbed material voided and increases stool output.		Secondary bile acids and hydroxy fatty acids may increase risk of colonic disease. They stimulate colonic motility and secretion.

### 1.9.2 Digestion in the infant

In the infant, adequate digestive function is necessary for normal growth and development. Although information regarding functional development of the gastrointestinal tract in humans is limited, it is known that some gastrointestinal functions are less well-developed in infancy. Development of the gastrointestinal tract begins in utero and continues for several months after birth. The factors necessary for digestion are shown in Table 1.13.

<b>Table 1.13                      Factors necessary for digestion that are present at birth.</b>	
<b>Factors</b>	<b>% of Adult</b>
Pancreatic alpha amylase	0
Salivary alpha amylase	10
Lactase	>100
Sucrase-isomaltase	10
Glucoamylase	<100

Functional development appears to be very limited prior to 26 weeks (Lebenthal, 1983) and some functions develop faster than others, which may have particular implications for premature infants. Functional development is summarised in Table 1.14.

#### 1.9.2.1 Suckling and Swallowing

There are three stages of development of sucking to allow the intake of food in the neonate. Initially the mouth is just making motions which does not result in effective sucking. This is followed by an 'immature suck-swallow' action where bursts of 4-7 sucks are seen but these are not followed by swallows, finally the mature mechanism comes in to force whereby 30 or more sucks are seen followed by swallowing every 1-4 sucks. The mature pattern is associated with propulsive peristaltic waves in the oesophagus and is seen a few days after birth in term infants (Guandalini, 1991). The acceptance of solid foods into the mouth does not

occur until later in post-natal development, it is not until 4-5 months that the infant is able to depress the tongue to allow the transport of food to the back of the mouth for swallowing (Guandalini, 1991). Contraction of the oesophageal muscles to allow propulsion of food to the stomach is present at birth.

**Table 1.14 Development of gastrointestinal tract in human fetus. First appearance of developmental markers.**

<b>Anatomic</b>		<b>Weeks gestation</b>
Oesophagus	Superficial glands develop	20
	Squamous cells appear	28
Stomach	Gastric glands form	14
	Pylorus and fundus defined	14
Pancreas	Differentiation of endocrine and exocrine function	14
Liver	Lobules form	11
Small intestine	Crypt and villi develop	14
	Lymph nodes appear	14
Colon	Diameter increases	20
	Villi disappear	20
<b>Functional</b>		
Sucking and swallowing	Mouthing only	28
	Immature suck-swallow	33 to 36
Stomach	Gastric motility and secretion	20
Pancreas	Zymogen granules	20
Liver	Bile metabolism	11
	Bile secretion	22
Small intestine	Active transport of amino-acids	14
	Glucose transport	18
	Fatty absorption	24
Enzymes	Alpha glucosidases	10
	Dipeptidases	10
	Lactase	10
	Enterokinase	26

From Lebenthal, 1983

### **1.9.2.2 Salivary Glands and Tongue**

The role of the salivary glands in utero is not known and it is not clear whether complete function is present at birth, except in the case of salivary amylase. Human new-borns have an abundance of saliva (Lourie, 1943) but the resting salivary flow rate and characteristics of salivary proteins have not been accurately measured. Parotid secretion is eight times higher in children aged 3-4 years compared with children age 10 and over (Lourie, 1943), suggesting very high flow rates in infants. Mixed salivary chloride falls markedly during the first week of life and then more slowly to a plateau at age 2 years. Salivary IgA is undetectable until 10-12 days although the epithelial component of secretory IgA is present at birth. Salivary IgA levels rise by 2 months of age but do not reach adults levels until mid childhood (South, 1971). IgG and albumin is found in samples up to 10 days but not after that. As the pH of the mouth is near neutral salivary amylase hydrolyses starch but food remains in the mouth so briefly the amount hydrolysed is low (Davenport, 1968).

### **1.9.2.3 Gastric Function**

The stomach capacity at birth is 10-12ml but increases to 200ml in the first 12 months. New-borns require small, frequent feeds as the stomach empties in 2.5-3 hours. As the infant matures the emptying rate depends on the amount and composition of food consumed. The stomach is slightly alkaline at birth, due to amniotic fluid, but within 24 hours gastric acid secretion is comparable to that of a three-year-old child. It then declines to a level lower than that of an adult for the first few months (Lebenthal *et al.*, 1983).

The proximal stomach relaxes in response to swallowing and so takes in the ingested food (Milla, 1986). Solids are retained in the stomach until the particles are no bigger than 1mm in size (Guandalini, 1991). The gastric parietal cell mass is well developed but the secretion of hydrochloric acid and pepsin per unit body weight is less for the infant than it is for the adult (Agunod *et al.*, 1969). Gastric

juice has lipolytic activity and, unlike pancreatic lipase, bile acids are not needed for hydrolysis of milk particle triglyceride (Cohen *et al.*, 1971). Peptic activity is found in the fetal stomach at 16 weeks and increases markedly between 28 and 40 weeks. After birth, pepsin levels reach adult levels by eighteen months (Schmitz and McNeish, 1987). Levels of plasma pepsinogen are 1.5 times higher than in older children and adults (Grayzel *et al.*, 1962). Table 1.15 shows the major proteases and their availability in the new-born.

Emptying of the gastric contents into the duodenum is mainly controlled by osmoreceptors in the duodenum. These osmoreceptors seem to function in the first few days of life (Hunt, 1961; Husband *et al.*, 1970). In the fasting state, there is a highly propulsive migrating motor complex, which moves from duodenum to ileum. When food enters the duodenum, this activity is disrupted and replaced with a segmenting and mixing activity that facilitates digestion and absorption of intestinal contents (Guandalini, 1991). By 34-36 weeks, the migrating motor complex is mature, at the same time there appears to be an irregular segmenting and mixing activity. When weaning occurs, the humoral messengers and receptors that allow postprandial activity to be fully expressed mature resulting in a more adult handling of intestinal content (Guandalini, 1991).

#### **1.9.2.4 Small Intestine**

Secretion of pancreatic juice and bile is stimulated by entry of food into the duodenum. Some absorption occurs in the duodenum but most takes place more distally in the upper jejunum. Activities of the two main cytosolic enzymes - glycyl-L-leucine peptidase and imidopeptidase - are present the fetus at 11-14 weeks and do not increase further with gestational age. Lysosomal activities are also present at 10 weeks of gestation and remain stable throughout gestation at a level comparable to that of infants (Schmitz and McNeish, 1987).

Lipase and trypsin levels, detectable in tissue homogenates of pancreas at 3 months gestation, remain extremely low until the end of the second trimester



Schmitz and McNeish, 1987). Chymotrypsin is not detected. It is only during the last trimester that enzyme activities rise. Proteolytic activity and enterokinase (which activates trypsin) is detectable at 26 weeks whereas amylase is not seen during gestation (Track *et al.*, 1975).

**Table 1.15 Proteases and their availability in the new-born**

Enzyme	Origin	Site of action	Type of enzyme	Activity in the new-born
Pepsins	Stomach	Stomach	Aspartic acid	Trace to none
Chymosin	Stomach	Stomach	Aspartic acid	Adequate
Enteropeptidase	Intestine	Intestinal lumen	Endopeptidase	Adequate
Trypsins	Pancreas	Intestinal lumen	Endopeptidase (serine)	Adequate
Chymotrypsins	Pancreas	Intestinal lumen	Endopeptidase (serine)	Adequate
Elastases	Pancreas	Intestinal lumen	Endopeptidase (serine)	Low
Carboxypeptidase (A and B)	Pancreas	Intestinal lumen	Exopeptidase (metallo enzymes)	Adequate

From Hamosh, 1996

At birth and in the first month of life pancreatic trypsin, chymotrypsin, carboxypeptidase B (see Table 1.15) and lipase activities are fairly well developed and increase to soon reach adult levels (Zoppi *et al.*, 1972).  $\alpha$ -Amylase is still not detectable at birth but slowly increases until 3-5 years of age (Rodgers *et al.*, 1974). The increasing digestive and absorptive capacities seen in the first years of life are due to increased maximal secretion, increased absorptive and digestive surface and not due to more enzymes (Schmitz and McNeish, 1987). Bile salt concentration is very low at birth and there may also be a difference in bile salt composition (Poley *et al.*, 1964). Critical micellar concentration may not be achieved which will severely reduce fat absorption capacity. It is for this reason that medium chain triglycerides are included in modern formula as these are not dependent on micelle formation for their absorption (Goedhart and Bindels, 1994). Lactase activity of full term infants is high at birth and remains so

throughout infancy. However by eight years of age lactose intolerance levels are the same as in an adult (Paige *et al.*, 1972).

#### **1.9.2.5 Colonic Function**

The colon develops in the embryo at the same rate as the small intestine (Grand *et al.*, 1976). The colon can be considered as an organ of conservation and may be a determinant (along with other factors) in occurrence of diarrhoea. Colonic transport has not been studied to any great extent in the infant. Younaszai *et al.*, 1978 showed by rectal dialysis studies that electrogenic Na<sup>+</sup> absorption and anion exchange are not well developed at birth but develops rapidly during the first year of life. The development of the functions dependent on the microflora will be discussed in a later section.

#### **1.9.3 Development of carbohydrate digestion**

In infants approximately 40% of the energy intake is from carbohydrates (Kien, 1996). Lactose is the major carbohydrate in new-borns fed solely milk (either breast or bottle) with small amounts of oligosaccharides also reaching the colon. New-born infants ingesting 120 kcal/ kg/ d consume approximately 12.6 g/ kg/ day of carbohydrate that must be digested and absorbed to provide an efficient energy source (Kien *et al.*, 1989). Dietary carbohydrates are digested and absorbed by the action of luminal and mucosal hydrolases and brush border proteins (Kein *et al.*, 1989).

Sugars are well utilised in the full-term infants. Maltase, isomaltase and sucrase activities reach adult levels by 28-32 weeks gestation. Lactase, present in low levels at 28 weeks gestation, increases near term and reaches adult levels at birth (Auricchio *et al.*, 1965) and its activity is high soon after birth (Lebenthal *et al.*, 1975). Complex carbohydrates are harder to digest in infancy due to the lack of necessary enzymes. Pancreatic amylases are low or absent up to 4 months of age and only reach significant levels of activity at about 1 year of age (Zoppi *et al.*, 1972). At this age therefore, salivary amylase and breast milk amylase are more

important. Salivary amylase, present at birth, rises to adult concentrations between 6 months and one year (South, 1971). A large amount of salivary amylase may be inactivated by hydrochloric acid in the stomach but infants do hydrolyse some starch. There is some evidence (Hodge *et al.*, 1983) that salivary amylase remains active in the stomach of the new-born allowing starch digestion to occur. This is thought to be due to the presence of glycosidase and  $\alpha$ -glucoamylase present in the brush border of the small intestinal enterocytes (Guandalini, 1991). These enzymes are active within the first few days of life (Lebenthal and Lee, 1980). They are able to split the glucose from the terminal end of amylose and amylopectin, especially those containing 5-9 residues of glucose. Longer polymers such as those found in starch are more difficult to digest in the first two months of life.

Carbohydrate that is not absorbed and digested in the small intestine then passes through to the colon. The carbohydrate is then available for fermentation by the colonic microflora and the subsequent production of SCFA is a mechanism of energy salvage as discussed earlier in this chapter.

## 1.10 DEVELOPMENT OF THE COLONIC FLORA

### 1.10.1 Effect of infant feeding practice on faecal flora

**Table 1.16 Differences in faecal flora of infants fed breast and formula milk**

Characteristics	Dietary Regime	
	Mother's milk	Formula milk
Bifidobacteria	10.7	10.0
Bacteroidaceae	6.1	9.9
Enterobacteriaceae	8.9	9.5
Streptococci	7.9	9.8
Lactobacilli	6.8	7.2
Staphylococci	5.8	5.5
Eubacteria	3.1	7.3
Peptococaceae	2.4	7.9
Clostridia	2.3	7.3
Veillonella	5.8	5.9

From Yuhura *et al.*, 1983 (Figures given are mean of log<sub>10</sub> counts)

It is now well established that the microflora in infancy is dependent on initial feeding practice. The differences between breast-fed infants and formula-fed infants in terms of their micro-ecological characteristics are summarised in Table 1.16 which gives mean  $\log_{10}$  counts of bacteria in infant faeces. In comparison, Figure 1.4 (page 53) gives patterns of bacterial dominance in infant faecal samples.

As long ago as 1899 it was found that gram-positive rods, mainly bifidobacteria were the predominant flora in breast-fed infants (Tissier, 1900). More recent studies have shown the flora of formula-fed infants is, by contrast, dominated by *E.coli* and gram negative anaerobic rods, mainly *Bacteroides* (Evaldson, 1982). In classic studies, Lejeune *et al.*, (1984) showed that in breast-fed infants, *B. bifidum* dominated and there was a suppression of gram negative bacteria whilst in formula-fed infants bacteroides were found to dominate and there were also high levels of enterobacteria. Other studies have confirmed this (Balmer and Wharton, 1989; Edwards *et al.*, 1994). Stark and Lee, 1984, saw that at four weeks of age the breast-fed infants had a simple flora consisting of bifidobacteria and lactobacilli with relatively few enterobacteria and enterococci and that colonisation by bifidobacteria was generally delayed in formula-fed infants.

However, some studies have failed to find a difference between the flora of the breast fed and formula fed infants. Lundequist *et al.*, 1985, examined the faecal flora of new-borns. Fifteen breast-fed and seven formula-fed were followed from birth to eight weeks and the composition of the faecal flora was investigated. Their study showed that bacteroides strains were the predominant anaerobes in both feeding groups whilst bifidobacteria occurred in less than half the specimens from both groups. Similarly, Simhon *et al.*, 1982, studied 15 breast-fed infants and 11 formula-fed infants and found that both groups had comparable flora predominantly gram negative rods with coliforms the dominant bacteria. Rose, 1984, and Gothefors, 1989, found that both breast-fed and formula-fed infants were dominated by *E.coli* and bacteroides followed by clostridium and bifidobacterium. These studies showing no differences between breast-fed and

formula-fed infants have been carried out in London, Stockholm and Scotland, therefore it cannot be seen as a country effect. The origin of bifidobacteria is obscure and Holdeman *et al.* (1977) reported that they were sometimes in the vagina of pregnant women. One possible explanation for this is the increasing use of vaginal antiseptic and increasing hygienic practices which may reduce the chances of an infant acquiring the bifidobacteria from its mother.

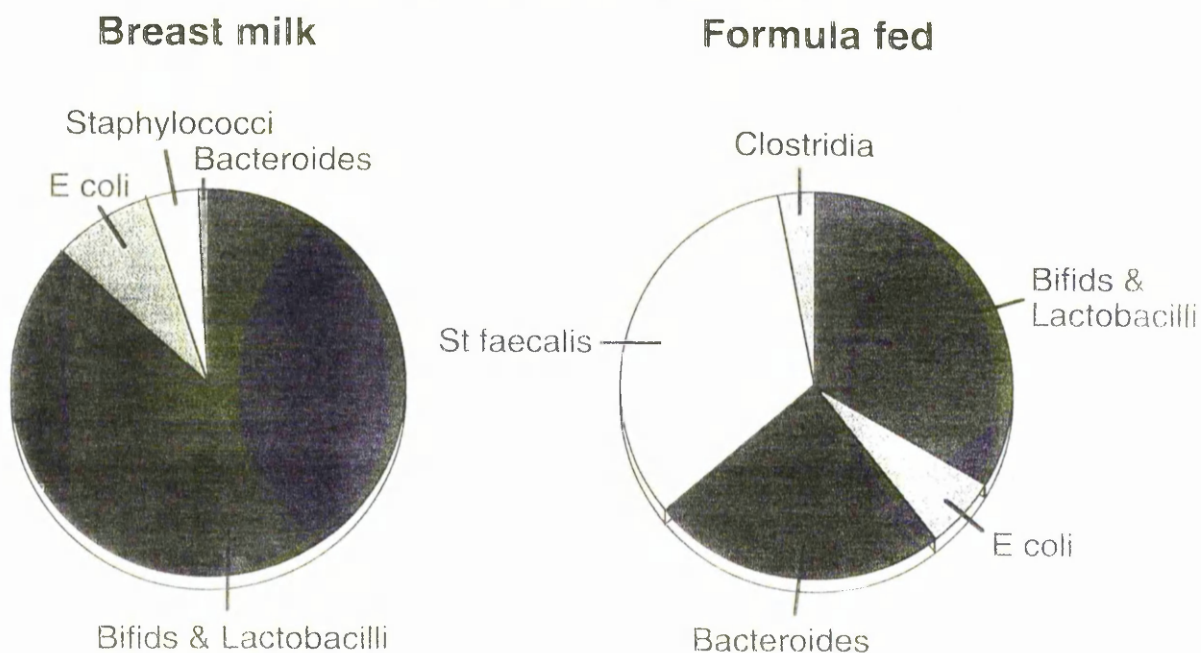
#### **1.10.2 Initial colonisation**

Gestational age, mode of delivery and type of feeding can all have an effect on the faecal flora of infants (Long and Swenson, 1977). It has been suggested that infants born vaginally obtain their gut flora from maternal vaginal and faecal flora (Tannock *et al.*, 1990). For infants born by caesarean section and infants separated from their mothers for long periods after birth, the environment becomes increasingly important in colonisation. Grönlund *et al.*, (1997) compared 30 infants delivered by caesarean section with 34 delivered vaginally. The colonisation of infants born by caesarean section was delayed. Bifidobacterium colonisation rates reached that of vaginally delivered infants at 1 month and lactobacilli colonisation rates at 10 days. It was suggested that these differences could be due to the administration of ampicillin before section. Ampicillin is known to alter colonic flora and, because it crosses the placenta readily, maternal and fetal ampicillin levels equilibrate within 1 hour (Bray *et al.*, 1966). However, ampicillin is thought to be only one of the factors causing the altered flora patterns. These differences in gut flora may last for up to six months after birth (Grönlund *et al.*, 1999) and the clinical relevance of these alterations is as yet unknown.

During the first 12 hours of life in both breast-fed and formula-fed infants, the dominant species in the meconium are facultative anaerobes such as Enterobacteriaceae (mainly *E. coli*) and streptococci. These rapidly increase to a population of  $10^5$  -  $10^{10}$  bacteria/ g of wet faeces by 48 hours (Schmitz and McNeish, 1987). At this stage, differences in the flora of the two feeding groups

appear (Schmitz and McNeish, 1987). In breast-fed infants, the facultative anaerobes promote a reduced environment that promotes the growth of bifidobacteria (a strict anaerobe). At the same time, the facultative anaerobes decrease in number. In contrast, formula-fed infants have a similar increase in bifidobacteria but without the decrease in facultative anaerobes  $10^6$ - $10^8$  bacteria/ g wet weight of faeces (Schmitz and McNeish, 1987; Fuller, 1991). This gives the classic profile of breast-fed infants: a faecal flora dominated by bifidobacteria and lactobacilli (Figure 1.4). Formula-fed infants have less bifidobacteria and lactobacilli, and more bacteroides, enterobacteriaceae and streptococci (Stark and Lee, 1982; Balmer and Wharton, 1989; Edwards *et al.*, 1994). At this stage, the flora of the formula-fed infant is more diverse and is more like the flora of an adult. Very few studies have investigated infants that are fed a mixture of breast-milk and formula-feeds and consequently the faecal flora profile of this group of infants is unknown. There are a large number of mixed fed infants in Britain who are given a variety of proportions of breast milk and formula feed (see Table 1.10). This in itself makes this group very difficult to characterise in terms of bacterial profile. In the studies described in this thesis, the development of their fermentation capacity and faecal SCFA will be described for the first time.

Figure 1.4 Faecal flora of breast-fed and formula-fed infants  
Patterns of bacterial dominance in infant faecal samples



In the breast fed infant, facultative anaerobes increase and create a favourably reduced environment that allows the bifidobacteria to become the dominant species,  $10^{10}$ - $10^{11}$  bacteria/ g wet weight of faeces (Bullen *et al.*, 1976; Schmitz and McNeish, 1987). Once solid food is added to the diet the faecal flora begins to change rapidly. Facultative anaerobes are found at higher counts  $10^9$ - $10^{10}$ ,  $10^7$ - $10^9$  bacteria/ g wet weight of faeces (Enterobacteriaceae, streptococci respectively). Gradually the strict anaerobes increase and bacteroides become the dominant species  $10^{10}$ - $10^{11}$  bacteria/ g wet weight of faeces, with a decrease in the prominence of bifidobacteria. As the flora of the formula-fed infants is already more diverse before weaning, less changes are seen as solid food is introduced. By about two years of age the flora of both groups of infants is very similar and resembles the stable adult flora (Fuller, 1991).

Overall it has been suggested that introduction of either mixed feeding or solid food causes a major disturbance in the colonic bacterial flora of the breast-fed infant, the impact on formula-fed infants is not as great (Schmitz and McNeish, 1987). It may be at these two critical stages that the breast-fed infant is more at risk of developing gastrointestinal infections (Bullen *et al.*, 1977). Prior to weaning in infants a significant amount of dietary carbohydrate may escape digestion because of the lower activity of pancreatic and brush border enzymes (McClean and Weaver, 1993). This dietary carbohydrate is mainly lactose and fructo-oligosaccharides in breast-fed infants but other carbohydrates may be present in formula milk. As weaning begins infants are exposed for the first time to many complex carbohydrates including dietary fibre and starch. These carbohydrates may escape digestion because of lack of chewing ability and pancreatic exocrine function in these young children. These substrates will pass through to the colon where they may be anaerobically metabolised to produce SCFA and lactate and gases. Inefficient fermentation of unabsorbed carbohydrate may result in a greater tendency to diarrhoea and previous studies have shown that breast-fed infants are more susceptible to gastrointestinal infections during weaning (Gordon, 1971; Lifschitz, 1996).

The period of weaning has not been fully investigated but it is likely that as the infant comes into contact with new substrates the bacteria may change. Previous work in weanling mice (Lee and Gemmell, 1972) showed that ingestion of the first solid food corresponded with the appearance of strictly anaerobic fusiform bacilli in the colon alongside a 10,000-fold decrease in coliform bacilli. This was reflected in increased production of SCFA especially butyric acid. Work in rats (Armstrong *et al.*, 1992) found that what was fed at weaning had a significant effect on SCFA produced in later life. It is known that the difference in faecal flora in humans has disappeared by about the age of two years when the flora appears to become like that of the adult (Midtvedt and Midtvedt, 1992).

Although differences between breast-fed and formula-fed infants in terms of their faecal flora is well established very little is known about infants that receive a mixture of feeding. This mixture may be transfer to formula milk after a period of breast-feeding or a combination of breast milk and formula milk. It is not known whether this group has patterns like that of the breast-feeding or formula-feeding group. There has been a study to suggest that if one bottle of formula milk is given this causes the flora to change from being that of a breast-fed infant to that of a formula-fed infant (Bullen *et al.*, 1977).

### **1.11 DEVELOPMENT OF BACTERIAL METABOLIC ACTIVITY**

The bacterial flora has a wide range of possible metabolic activities. Various studies have been carried out in Sweden by Midtvedt and co-workers, which show the slow development of other metabolic activities (Midtvedt *et al.*, 1988; Midtvedt and Midtvedt, 1992; Midtvedt and Midtvedt, 1993; Midtvedt *et al.*, 1994). In these studies the establishment of five flora-related functions was investigated. These functions were: production of short chain fatty acids, degradation of mucin, conversion of bilirubin to urobilinogen, conversion of cholesterol to coprostanol and inactivation of faecal tryptic enzyme. These functions were investigated in seventeen healthy Swedish children from birth to six months. The infants were split into four groups depending on their intake of



breast-milk. Group I (n=1) received solely breast-milk for the six months of the study, group II (n=8) received exclusively breast-milk until three months of age, group III (n=2) received exclusively breast-milk until at least one month of age and group IV (n=6) who received formula supplement at one month of age.

The SCFA profile of the children suggested that the children who had a more mixed diet tended to have a more varied adult-like profile of SCFA. Those who received only breast milk produced fewer and shorter chain fatty acids. Interestingly, the infant who had received solely breast-milk until six months of age produced only three SCFA at this age; acetic acid 96.3%, propionic acid 2.2% and n-butyric acid 1.5%. This is in contrast to work by Edwards *et al.*, (1994), that showed breast-fed infants usually had a SCFA profile with high acetate and lactate, however, lactate was not measured in the study of Midtvedt and Midtvedt (1988). Production of SCFA was the first function to be established. This followed by the conversion of bilirubin to urobilinogen. At six months, however, the average value of urobilinogen was still only about a tenth of values found in adults. In four of the subjects urobilinogen production was absent. This was consistent with another investigation where it was found to be absent in one of ten children at two years of age (Norin *et al.*, 1985).

Mucin is assumed to be utilised by the intestinal flora. Norin *et al.*, 1985 showed that in very young infants (up to 5 days of age) a germ-free animal characteristic pattern was seen. Microbial mucin degradation did not occur. In older infants, 20 of 46 samples from 23 infants aged 2-11 months and in all samples of children over 20 months there was a shift to a microflora-associated characteristic. The establishment of a mucin degrading flora occurred at approximately 3 months of age (Midtvedt *et al.*, 1988; Midtvedt *et al.*, 1994).

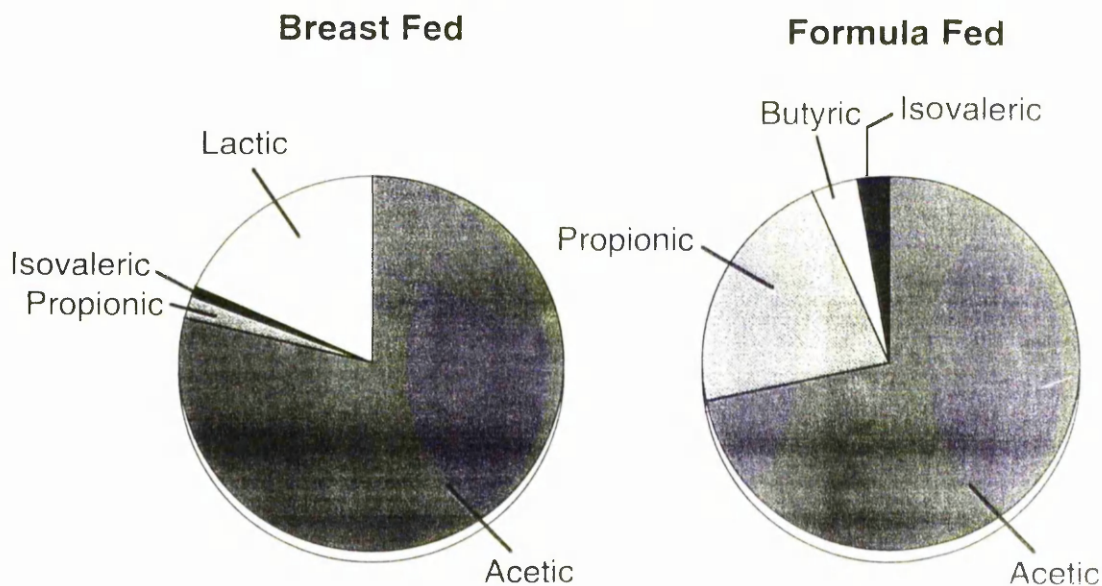
These researchers also studied faecal tryptic activity. Faecal tryptic activity can be defined as the sum of several complex interactions. These include secretion of trypsinogen and trypsin inactivators from the pancreas, activation of trypsinogen by intestinal brush border enzymes, intestinal presence of microbial and dietary

derived compounds that inactivate and degrade trypsin and its inactivators. The values of infant faecal tryptic activity were found to be lower than in adults. No conversion of cholesterol to coprostanol was seen in any of the samples. The overall conclusion of this study was that the development of a fully functional active flora is a slow process but the maturation of the system varied. Some activities were almost fully established before others had started to develop.

#### **1.11.1 SCFA Production in infants**

In the normal neonate and infant, the SCFA production changes as the colonisation of the digestive tract occurs. In the meconium, SCFA concentrations are very low, only about 10 % of the adult values. They increase significantly in the first 4 days of life; however, the proportions of individual SCFA are different from adults. There is a higher proportion of acetate and lactate in the faeces of infants, and butyrate production that is thought to be important in adults is very low. The ratio of acetate/propionate/butyrate/other acids at 4 days was 89/5/5/1 in infants. This probably reflects not only differences in flora but also differences in carbohydrates available for fermentation. The primary carbohydrate for the colonic flora of neonates is lactose and this may account for the high proportion of acetate. The profiles of SCFA depend on whether the child is breast or formula fed. Previous work (Edwards *et al.*, 1994) has shown that in pre-weaned infants there are marked differences in the patterns of SCFA relating to the differences in microflora found in breast-fed and formula-fed neonates. The breast-fed infant with predominantly bifidobacteria and lactobacilli produces mainly acetic acid and lactic acid (Figure 1.5). In contrast, the more diverse flora of the formula-fed infant produces mainly acetic acid and propionic acid with a small amount of butyric acid and no or little lactic acid. Again, in this respect the formula-fed infants are more like the adults. The concentrations and profiles of SCFA in infant faeces change as weaning progresses (Midtvedt and Midtvedt, 1992).

Figure 1.5 Faecal SCFA in breast-fed and formula-fed infants



The development of the colonic flora is very slow and there appears to be potential for manipulating it at this early stage of life.

#### 1.12 CHANGES IN CHARACTERISTICS OF FAECES COLOUR ODOUR CONSISTENCY

The faeces of breast-fed and formula-fed infants differ in colour, odour and consistency; however, very few studies have investigated these characteristics. Breast-fed infants have more liquid faeces with a 'cheesy' odour and an acid pH of approximately 5.0 (Bullen and Willis, 1971). In contrast, the faeces of formula-fed infants are more adult-like in consistency, odour and pH (approximately 6.0-7.0).

Weaver *et al.*, 1988b, studied the stools of breast-fed and formula-fed infants prior to weaning and the effect addition of solids to the diets had on the stools. Although the stools of all infants were almost uniformly yellow at eight weeks of age, about 30% of the stools of formula-fed infants changed to a green colour.

The introduction of solid foods during weaning was associated with a change from yellow to brown in both feeding groups. As none of the infants received solids before 8 weeks, the change in colour of the stools of formula-fed infants could not be related to addition of solids.

Prior to weaning, the stools of breast-fed infants were significantly larger than those of formula fed infants, but upon introduction of solids, this difference disappeared. The consistency of stools changed from soft to hard with age in both feeding groups. Throughout the first 20 weeks, the breast-fed infants produced softer stools both prior to and after weaning. Lemoh and Brooke, (1979), reported a rise in faecal weight of formula-fed infants from 11-35 g during the first two years of life but this was not associated with a change in water content. The frequency of bowel movements was much higher in breast-fed infants during the first 8 weeks of life. By sixteen weeks, both groups had two bowel actions per day.

Weaver and Lucas, (1993), studied pre-term infants, a group fed exclusively breast milk (mother's own, donated or a combination) and those fed formula milk (term or pre-term). Again as with the term infants, those who received human milk had a greater defaecation rate than those on formula feeds. The consistency of stools in human milk fed preterms was significantly softer than those of the formula-fed infants and this difference persisted throughout the study period of 56 days.

Bowel habit is function of dietary composition and the passage through the gastrointestinal tract. The decline in frequency and softness of stools with increasing age is probably due to maturation of the water conserving capacity. Breast milk contains non-nutritive proteins and oligosaccharides that escape digestion and absorption. This may cause the greater stool volume in breast-fed infants if they are not fermented to SCFA, which are then absorbed in the colon. Quinlan *et al.*, (1995), showed marked differences in composition of stools between breast-fed and formula-fed infants. The stools of formula-fed infants had

higher levels of minerals and lipids than those of breast-fed infants. The differences in lipid content was mainly due to fatty acids, especially c16:0 and C18:0. This may be indicative of differences in the large bowel and it has been reported that the gut flora influences saturated fatty acid absorption (Combe *et al.*, 1976).

### **1.13 FACTORS AFFECTING BACTERIAL COLONISATION**

Many factors, environment, diet, genetics, may influence the colonisation of the neonatal gut. The exact role of each factor is difficult to investigate in isolation *in vivo*. Modern formula feeds try to mimic all components of human milk, as closely as possible. However, there still remain differences between the flora of breast-fed infants and formula-fed infants (Balmer and Wharton 1989).

#### **1.13.1 Carbohydrate source**

Carbohydrates contribute about 40% of the energy intake of breast-fed or formula-fed infants. The carbohydrate intake may have a significant role in the development of the bacterial flora and its consequent actions. Lactose, a disaccharide composed of glucose and galactose, is the major carbohydrate in human milk (Kien, 1996). The lactose content of mature human milk varies between 6.8 g/ dl and & 7.4g/ dl (Committee on the Nutrition of the Pre-term Infant, European Society of Paediatric Gastroenterology and Nutrition).

Chromatographic analysis of human milk has shown trace amounts of glucose, galactose and medium chain length carbohydrates (oligosaccharides). Small amounts of oligosaccharides can also reach the large intestine. However, lactose is most significant in determining the bacterial characteristics. There are two major fates of dietary lactose in the newborn. It may be digested in the small intestine to give glucose and galactose that are then absorbed. The utilisation of lactose depends on its hydrolysis by lactase, a disaccharidase found in high quantities in infancy. Any undigested lactose will travel through to the colon

where it is fermented to produce short chain fatty acids and gases. The main SCFA produced are acetic acid and lactic acid. Propionate is only found in small amounts in infant faeces and virtually no butyrate is found despite its importance in adults (Roediger, 1980; Kruh, 1982; Scheppach *et al.*, 1992; Weaver *et al.*, 1988a).

It has been hypothesised that there may also be a bifidus factor that actively promotes the growth of bifidobacteria (Beerens *et al.*, 1980). Fresh human milk was shown to promote the growth of *B. bifidum*, *B. longum* and *B. infantis*. Various workers have put forward possible candidates for the bifidus factor, i.e. growth factors for bifidobacteria:

Lactulose was suggested as a growth factor by Gedek, (1968). This study also proposed that amino sugars and glycopeptides were involved in promoting bifidobacteria. However, this seems unlikely, as lactulose does not occur naturally in human milk.

Derivatives of pantothenic acid may act as a growth factor for *Bifidobacterium infantis* (Tamura *et al.*, 1972).

Oligo- and polysaccharides used specifically by bifidobacterium were suggested by Yazawa *et al.*, 1978. Neut *et al.*, 1980 also suggested that an oligosaccharide is involved in promoting the growth of bifidobacterium. Certain oligosaccharides promote the growth of lactobacilli in the lower intestinal tract. As a consequence acetic acid is produced by these bacteria inhibiting the multiplication of bacterial pathogens.

Bovine casein isolates were shown to promote the growth of a strain of *Bifidobacterium bifidum* var. *pennsylvanicus* by Kehagias and co-workers in 1977.

### **1.13.2 Buffering capacity**

The faecal pH of breast-fed infants is lower than that of formula-fed infants (pH 5.0, pH 6.0-7.0 respectively). This is one of the protective barriers against pathogens. Some bacteria are very pH sensitive, bifidobacteria and lactobacilli prefer acidic conditions. Acetic and lactic acid are produced and the low pH (pH 5.0) favours the growth of these organisms rather than the bacteroides. The low phosphate and casein content of breast milk also maintains this low pH. This accounts for the acidic faeces found in breast-fed infants. In formula-fed infants, the acid end products do not accumulate and so the bifidobacteria and lactobacilli are less able to thrive and other bacteria can compete against them.

### **1.13.3 Protein digestibility**

Casein/whey predominance was shown to have an effect on the flora. Whey protein predominant formula produced higher amounts of bifidobacteria and less bacteroides species than casein protein predominant formula. The low phosphate and casein content of breast milk and whey predominant formulas limit the buffering capacity so a low pH is maintained. In these conditions favouring the growth of bifidobacteria whilst inhibiting the growth of bacteroides (Balmer *et al.*, 1989).

### **1.13.4 Iron and lactoferrin**

Many bacteria need a source of iron. Lactic acid bacteria do not. A restriction on the availability of iron in the gut would favour lactobacilli and bifidobacteria (Archibald, 1983). The availability of iron in the large intestine is dependent on the amount of iron that escapes absorption in the small intestine, which in turn is dependent on the amount in the diet, the efficiency of the absorption process and the presence of any iron chelating agents.

Iron is not needed in significant amounts by bifidobacteria and lactobacilli for growth (Archibald, 1983) but it is necessary for the growth of potential pathogens. Human milk contains less iron than modern milk formula but breast-fed infants are more efficient at absorbing it. Lactoferrin is present in significant amounts in human milk, 1.5g/ l (Rueda and Gil, 2000). It binds iron therefore making unobtainable for growth by pathogens and allows the bifidobacteria/lactobacilli to thrive. Lactoferrin has a role against infection and immunotropic properties (Adamic and Wlaszczyk, 1996). It is also a growth-promoting factor for human B and T lymphocytes (Hashizume *et al.*, 1983) and may promote the viability of gut derived lymphocytes and human milk lymphocytes. In experiments by Balmer and Wharton (1991), iron mixed with both casein and whey formulae discouraged the growth of staphylococci and bacteroides but encouraged growth of clostridia and enterococci. The flora seemed to become more dissimilar to that of a breast-fed infant. Iron-free formula gave a profile that had some similarities to a breast-fed infant but this flora was still not dominated by bifidobacteria and lactobacilli. Lactoferrin also did not increase the growth of these organisms. It is thought that this could be due to several different reasons. Lactoferrin was inactivated when added to the formula; the bovine lactoferrin used may have attracted foreign protein response negating its effect; other factors necessary for its optimum activity were not present. Lactoferrin is one of the whey based proteins and this may be why whey based formulas gave a flora more similar to a breast-fed infant.

#### **1.13.5 Nucleotides**

Nucleotides are found in human milk in significant amounts (Gil and Sanchez-Medina, 1982) but only small quantities are present in formula feeds. Nucleotides have been thought to enhance functions such as cellular immunity (Carver *et al.*, 1991) and lipid metabolism (Sanchez-Pozo *et al.*, 1994). In addition, other in vitro studies have shown that nucleotides act as growth factors for some strains of bifidobacteria. Nucleotides could be linked to increasing iron absorption in the small intestine (Balmer and Wharton, 1991). Whilst some studies have shown that addition of nucleotides to infant formulas created a faecal flora more similar



to breast-fed infants (Gil *et al.*, 1986), others have suggested there has been no effect (Balmer *et al.*, 1994).

#### **1.14 DEVELOPMENT OF FERMENTATION CAPACITY IN INFANCY**

Carbohydrate fermentation has been investigated considerably in adults but information about the colonic flora and development of fermentation capacity in infancy and childhood is limited. Although there is some information of infant faecal SCFA, these may not reflect true bacterial activity as they are the net result of both production and absorption and therefore provide little information about fermentation capacity. *In vitro* fermentation models, as used in this thesis, provide more information about SCFA production and the ability of the flora to ferment specific substrates.

Previous work has investigated fermentation in a small number of exclusively breast-fed (n = 14) and formula-fed infants (n = 9) before weaning (Lifschitz *et al.*, 1990). In their study, the rate and pattern of fermentation of lactose was compared between the two feeding groups at pH 5.5 and pH 6.8. In addition, lactose carbon hydrolysed was measured by calculating the amount of lactose remaining after incubation. At low pH the faecal cultures of breast-fed infants showed less hydrolysis of lactose into monosaccharides than formula-fed infants. However, in the formula-fed infants there was decreased fermentation. This resulted in increased osmolality due to the unfermented monosaccharides, if this were to occur *in vivo* this would increase the infants risk of diarrhoea. The Lifschitz model used an incubation time of one hour, but the transit time through the colon and therefore time available for fermentation would be much greater than this. As a consequence differences in lactose hydrolysis may be compensated before any effect in stool output would be seen. In addition, the faecal flora of formula-fed infants produced significantly more propionic acid in the cultures. There was also higher acetate (although this was not statistically significant) and total SCFA (significant at higher pH only) and no difference in lactate.

The fermentation of raw and cooked maize starch fermentation has been compared in infants, toddlers and adults (Christian *et al.*, 2000). The infant group related to late weaning (approximately 9 months) and the toddler group to very late weaning (19 months). The study was of cross-sectional design. At four hours incubation, infants produced more total SCFA with both types of starch than adults. Values for toddlers were comparable to adults for cooked maize starch but higher for raw maize starch. By 24 hours incubation, total SCFA was similar for infants and adults but less for toddlers with both raw and cooked maize starch. This coupled with the fact that the infants and toddlers produced more lactate at 4 hours suggested that adults were fermenting the starch at a slower rate. The authors proposed that maize starch (with high resistance to digestion) was present in the infant diet and therefore would pass undigested into the colon. This would allow the flora to adapt so that there was greater fermentation capacity for maize starch. The efficiency of these young children in fermenting starch leads to the potential for energy salvage when the digestive capacity of the small intestine is not fully developed. The numbers in this study, however, were very small ( $n = 6, 6, 7$ , for infants, toddlers, adults respectively) and greater numbers would be needed to verify these conclusions. The infants and toddlers were two different sets of children and it would be better to study the same children at each age to obtain a true picture of how the fermentation changes.

### **1.15 CONCLUSIONS AND AIMS OF THESIS**

There is much evidence that in infancy a significant amount of carbohydrate escapes digestion and absorption because of immature gut function. This carbohydrate enters the colon where it is available for anaerobic metabolism by the colonic microflora. However, there is a slow maturation of the colonic flora and fermentation capacity. This is supported by studies of that showed bacterial activities were still increasing up to two years of age (Midtvedt *et al.*, 1988) and studies showing that starch was present in the faeces of children up to 3 years of age (Verity and Edwards, 1994). Fermentation capacity of carbohydrate therefore may be important in the early stages of weaning. The studies of Lifschitz and

Christian suggest that the capacity to ferment carbohydrate may determine the energy salvage for food and may also affect stool output and faecal energy losses.

Given a paucity of information, the studies in this thesis were carried out to test the hypothesis that neonatal feeding practice determines the rate and pattern of development of, both the faecal SCFA and colonic fermentation capacity of carbohydrates during weaning.

It is well established that the faecal flora of breast-fed and formula-fed infants differs (Balmer and Wharton, 1989) and this is reflected in the profile of faecal SCFA produced (Edwards *et al.*, 1994) but changes through the weaning period have not been investigated. The results of a cross-sectional pilot study (described in Chapter 2) showed that there were differences in the fermentation capacity of breast and formula-fed infants, and that the development of fermentation capacity for complex carbohydrates was dependent on infant feeding practice. However the pilot study investigated small numbers of infants. To draw definitive conclusions a larger, longitudinal study was proposed. This study would follow infants from birth to allow investigation of weaning practice and development of fermentation capacity. This longitudinal study would also allow investigation of a wider range of carbohydrate substrates and samples would be collected up to two years of age. Given the large numbers of mothers in the UK who begin breast-feeding and then change to formula-feeding prior to weaning, either partially or completely, a group of infants termed 'mixed fed' were also studied.

## **Chapter 2**

# **Cross-sectional Pilot Study of Fermentation Capacity in Infancy**

## 2.1 INTRODUCTION

The main study described in this thesis was an extension of a pilot study (described here) based on faecal samples collected from a cross-section of breast-fed and formula-fed infants at different stages in the weaning period. The pilot study was undertaken because the factors that determine the bacterial colonisation and metabolic activities of the human colon are not well understood. Neonatal diet and the weaning period are thought to be critical factors in the initial colonisation of the gut and the establishment of the intestinal flora that will then remain stable throughout adult life. Carbohydrate fermentation has been thoroughly investigated in adults but knowledge of the colonic flora in neonates, and how it develops in response to new foods during weaning, is poorly understood.

It is well established that the breast-fed and formula-fed infants have different flora (Balmer and Wharton, 1989). Bifidobacteria and lactobacilli dominate the flora of breast-fed infants whereas the formula-fed infants have more bacteroides, enterobacteria and streptococci, which is more similar to the adult microflora. These differences in flora are reflected in the faecal SCFA, the products of bacterial carbohydrate fermentation (Edwards *et al.*, 1994).

After weaning, the microflora gradually develops to that of the adult, with more than 400 different bacterial species (Holdeman and Moore, 1974). Very little is known about the factors which influence these changes in microflora during weaning, but it is at this stage that the flora is developing in response to a wide range of new substrates including starch and dietary fibre. Any of these new substrates, including starch and dietary fibre that the neonate is unable to ferment would pass through the colon unmetabolised causing increased faecal output and possible diarrhoea. As discussed in Chapter 1, fermentation of carbohydrate produces short chain fatty acids (SCFA) which are absorbed, salvaging energy, and preventing osmotic water loss (Lifschitz, 1996). Weaning has been shown to

be the stage when diarrhoea is more likely to occur in breast-fed infants (Gordon *et al.*, 1971).

Very few studies have looked at the effect of weaning on the faecal flora. Many weaning foods contain complex carbohydrates and dietary fibres, so it is important to know how easily these will be fermented and the effect that these substrates have on the development of the colonic microflora.

Faecal SCFA profiles are the end result of both production and absorption and may not represent events in the colon where most fermentation occurs. They give very little information about the ability of infants to ferment carbohydrate. It is the ability of the colonic flora to ferment carbohydrate, and the absorption of SCFA, which helps prevent diarrhoea. It is difficult to study colonic fermentation *in vivo*. Successful *in vitro* studies have been carried out in adults where faecal bacteria were used to inoculate cultures containing different carbohydrates. Few studies of this kind have been carried out using faecal bacteria from infants.

The different colonic microflora of breast-fed and formula-fed infants is likely to affect their ability to ferment oligosaccharides and complex carbohydrates. In this cross-sectional pilot study, therefore, we used a simple *in vitro* method to test the hypothesis that the difference in the faecal flora of breast-fed and formula-fed infants influences their ability to ferment carbohydrates and that the ability to ferment different substrates changes as the weaning process progresses.

## **2.2 SUBJECTS AND METHODS**

### **2.2.1 Study design**

A cross-sectional design was used in this pilot study to test the hypothesis described above, in order that results could be obtained in a short space of time. Infants of different ages provided samples so that the different developmental stages could be investigated concurrently. This gave an indication of differences

in the fermentation capacity of the feeding groups but also allowed the practicalities of a larger, longitudinal study to be examined. Results from the pilot study were provided to allow a power calculation of subject numbers required to achieve statistically significant results in the larger, longitudinal study described in this thesis.

## 2.2.2 Subjects

In this cross-sectional study fifty-seven full-term infants were recruited by personal contact and advertisement. None of these babies, nor any of the breast feeding mothers, had received any antibiotics at the time of recruitment or at any time before giving samples.

Fresh faeces were obtained from 12 exclusively breast fed infants, 7 early weaned breast-fed infants and 8 late weaned breast-fed infants and these were compared to 10 exclusively formula fed babies, 10 early weaned formula-fed infants and 10 late weaned formula-fed infants. Early weaning was defined as being within six weeks of the first non-milk food, when liquidised or puréed food was given, and late weaning was defined as being after 16 weeks of weaning when chopped, solid food was ingested. Characteristics of the sample are given in Table 2.1.

Table 2.1	Description of subjects					
	Breast-fed infants			Formula-fed infants		
	PW	EW	LW	PW	EW	LW
Number	12	7	8	10	10	10
No of males	8	5	3	0	5	2
No of females	4	2	5	10	5	8
Median age (weeks)	5.5	22	32	6	19	37
Range	2-23	16-27	23-46	2-10	16-27	32-48

PW = Pre-weaning, EW = Early weaning, LW = Late weaning

Infants who were being fed a mixture of breast and formula milk were excluded from the study. The mothers were not influenced by the investigators in their

choice of formula feed. The formula fed babies in this study were receiving one of a range of different formula (Ostermilk, Farley's, UK; Ostermilk 2, Farley's, UK; SMA gold cap, Wyeth, U.S.A.; SMA white cap, Wyeth, U.S.A.).

### **2.2.3 Fermentation Model**

Fermentation capacity for carbohydrates was tested in an *in vitro* model modified from Adiotomre *et al.*, 1990. Fresh faecal samples were collected from the babies at their homes and were processed within an hour of defaecation. After weighing, a faecal slurry (32% w/v) was made with pre-reduced phosphate buffer pH 7. One ml of this was used to inoculate 9mls pre-reduced basic salts medium (Adiotomre *et al.*, 1990) containing 100mg of carbohydrate substrate; glucose (BDH, Loughborough, UK), raftilose™ (Raffinerie tirlémontoise SA), and soyabean polysaccharide (Scientific Hospital Supplies UK Ltd, Liverpool, UK). Cultures contained a total volume of 10ml. If less faecal material was available, amounts were halved to give a total culture volume of 5ml. Cultures were incubated in an anaerobic jar at 37°C for 24 hours. A control culture containing no carbohydrate was also incubated. Details of the fermentation method are given in Chapter 3.

### **2.2.4 Carbohydrate Substrates**

Substrates were chosen to reflect a range of simple and complex carbohydrates. Glucose is readily fermented by most bacteria and allows comparison of potential fermentation capacity more readily than do sugars or carbohydrates that need induction of enzymes. It is a major component sugar of many dietary fibres, resistant starch and maltodextrins. Raftilose™ was chosen as an example of an oligosaccharide, which may be present in significant quantities in breast milk. Soyabean polysaccharide (in soy flour) is used in weaning foods and is likely to reflect the sort of polysaccharides present in early and late weaning.



## **2.2.5 Measurements**

### **2.2.5.1 Culture Supernatant SCFA**

After 24 hours, the final pH of fermentation fluid was measured before storage at -20°C for later analysis of SCFA and lactic acid. SCFA were measured by GLC of acidified ether extracts using  $\beta$ -methyl valeric acid as internal standard (Spiller *et al.*, 1980). Lactic acid was measured similarly after methylation with sulphuric acid and methanol, using succinic acid as an internal standard (Holdeman and Moore, 1973). Full details of these methods are given in Chapter 3.

## **2.2.6 Statistics**

Fermentation data from breast-fed and formula-fed infants were compared with each other and against results from similar cultures containing adult faeces (n = 6). Comparisons were by Kruskal-Wallis followed by Mann Whitney U tests after subtraction of the no carbohydrate blank to allow for SCFA produced from other substrates present in the faeces.

## **2.2.7 Ethical Approval**

This study was approved by the ethics committee of Yorkhill NHS Trust. The mothers of all infants taking part gave written informed consent.

## **2.3 RESULTS**

### **2.3.1 pH values**

There were no significant differences in the final pH of fermentation cultures between the two infant feeding groups (Table 2.2). The greatest fall in pH after 24 hours incubation was observed for the sugars and fructo-oligosaccharides. The

final pH after 24 hour fermentation with soyabean polysaccharide was significantly higher ( $p<0.05$ ) for both feeding groups compared with adults.

**Table 2.2** Comparison of pH values after *in vitro* incubation of faecal bacteria from pre-weaned breast-fed infants and formula-fed infants, and adults in cultures containing simple and complex carbohydrates

	Breast-fed infants		Formula-fed infants		Adults	
	(n = 11)		(n = 11)		(n = 6)	
	Median	Range	Median	Range	Median	Range
Blank	7.0	5.5-7.6	7.0	6.8-7.5	7.1	7.0 -7.3
Glucose	4.3	3.5-6.3	5.1	3.6-6.3	4.9	4.3 -5.7
Lactose	4.3	3.5-5.8	5.0	3.7-6.3	ND	ND
Raftilose™	5.0	3.8-5.7	5.0	4.2-5.9	4.7	4.4 -5.4
Soyabean polysaccharide	6.7*	5.3-7.1	6.6*	6.3-7.0	6.3	5.9 -6.5

\*  $p<0.05$  compared with adults, ND = not done

### 2.3.2 Individual SCFA in culture supernatant

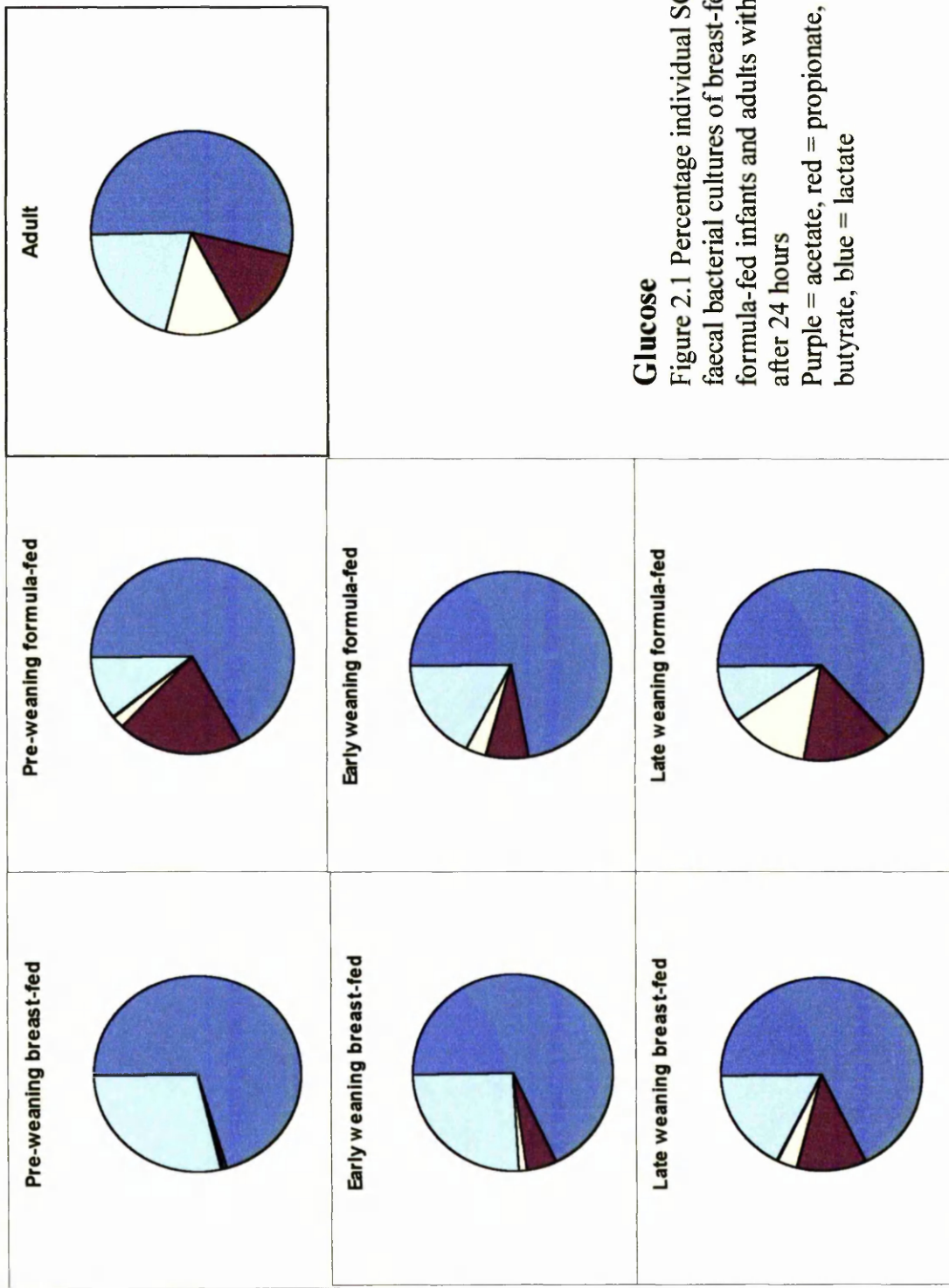
Comparisons of individual SCFA were made between breast-fed infants, formula-fed infants and adults, and also between pre-, early and late weaning for breast-fed and formula fed infants (Table 2.3). Both groups of infants differed significantly from the adults. At pre-weaning with glucose, adult butyrate concentrations were higher than for breast-fed and formula-fed infants ( $p<0.01$ ,  $p<0.05$ ). Propionate levels were higher in adults and formula-fed infants than breast-fed infants ( $p<0.001$ ). By early weaning, the only difference that remained was that adults still had higher levels of butyrate than breast-fed infants ( $p<0.05$ ). There were no differences at late weaning.

In cultures with raftilose™, the breast-fed infants had significantly less butyrate ( $p<0.05$ ) and significantly less lactate ( $p<0.05$ ) than adults. These differences had disappeared at early and late weaning.

Throughout all weaning stages when cultures were incubated with soyabean polysaccharide, less propionate was seen in the infants than in the adults ( $p<0.01$  at pre- and early weaning,  $p<0.05$  at late weaning). A similar picture was seen with butyrate where adults had higher levels than all weaning stages ( $p<0.05$ ). By late weaning the difference between adults and breast-fed infants was no longer apparent. However, breast-fed infant cultures had higher butyrate than formula-fed ( $p<0.05$ ) at late weaning. Acetate values were higher in adult cultures than those of breast-fed and formula-fed infants ( $p<0.05$ ) with soyabean polysaccharide but only at early weaning.

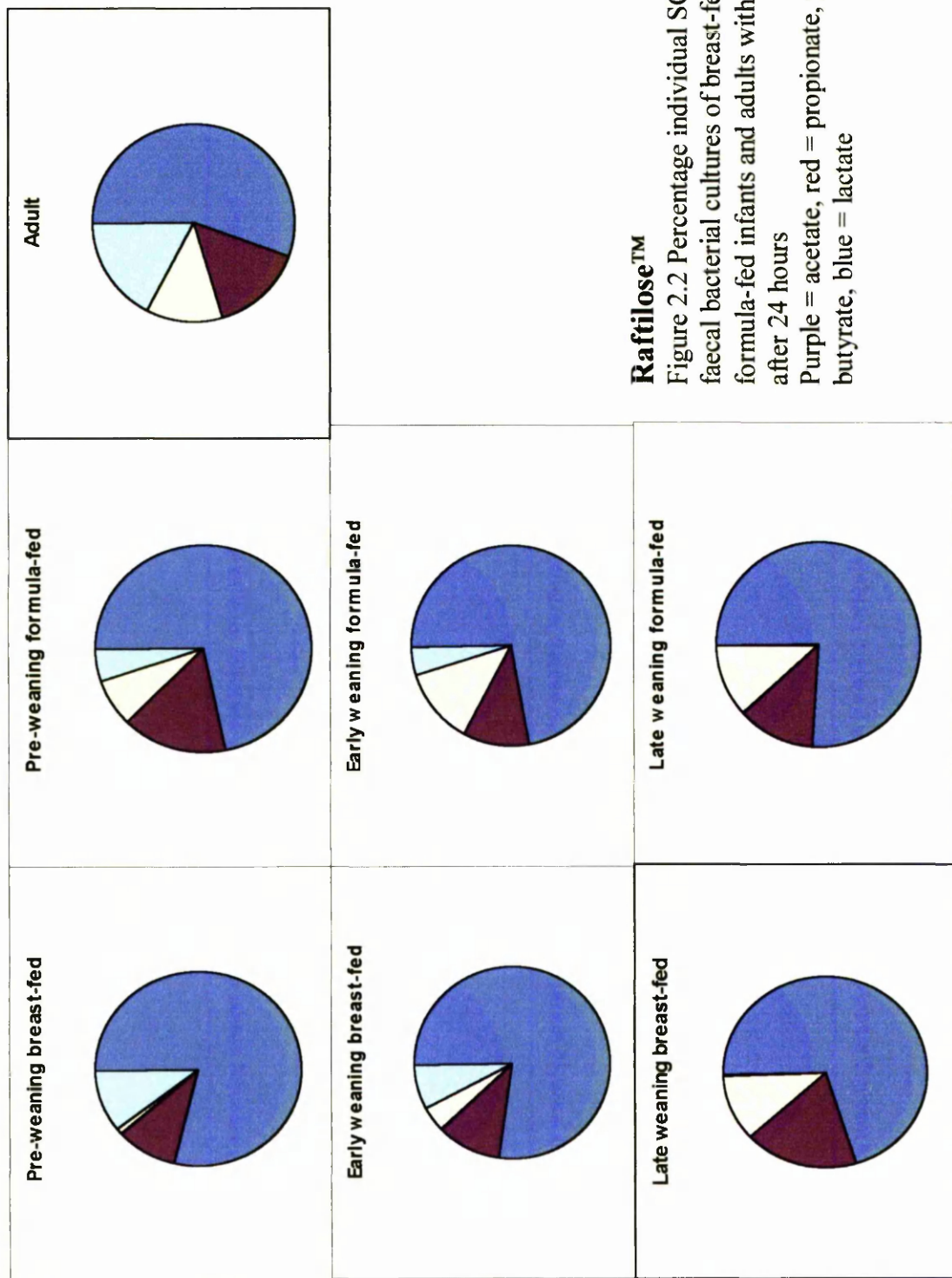
When comparing pre-, early and late weaning within each group of infants, no differences were seen in the formula-fed infants with any carbohydrate substrate. Significant differences between stages of weaning were seen in breast-fed cultures with all substrates. Pre-weaning values of propionate were lower than early ( $p<0.05$ ) and late weaning ( $p<0.01$ ) in cultures with glucose. Also with glucose there was lower butyrate at pre-weaning than at early weaning ( $p<0.01$ ). With *rafitilose*<sup>TM</sup> pre-weaning values were significantly lower ( $p<0.05$ ) than late weaning for acetate, propionate and butyrate. In cultures with soyabean polysaccharide butyrate concentrations at late weaning were higher than pre- ( $p<0.01$ ) and early weaning ( $p<0.05$ ). At pre-weaning and late weaning acetate values were higher than early weaning ( $p<0.05$ ).

Proportions of individual SCFA at each stage for breast-fed, formula-fed and adults are shown in Figure 2.1 (glucose), Figure 2.2 (*rafitilose*<sup>TM</sup>) and Figure 2.3 (soyabean polysaccharide).



### Glucose

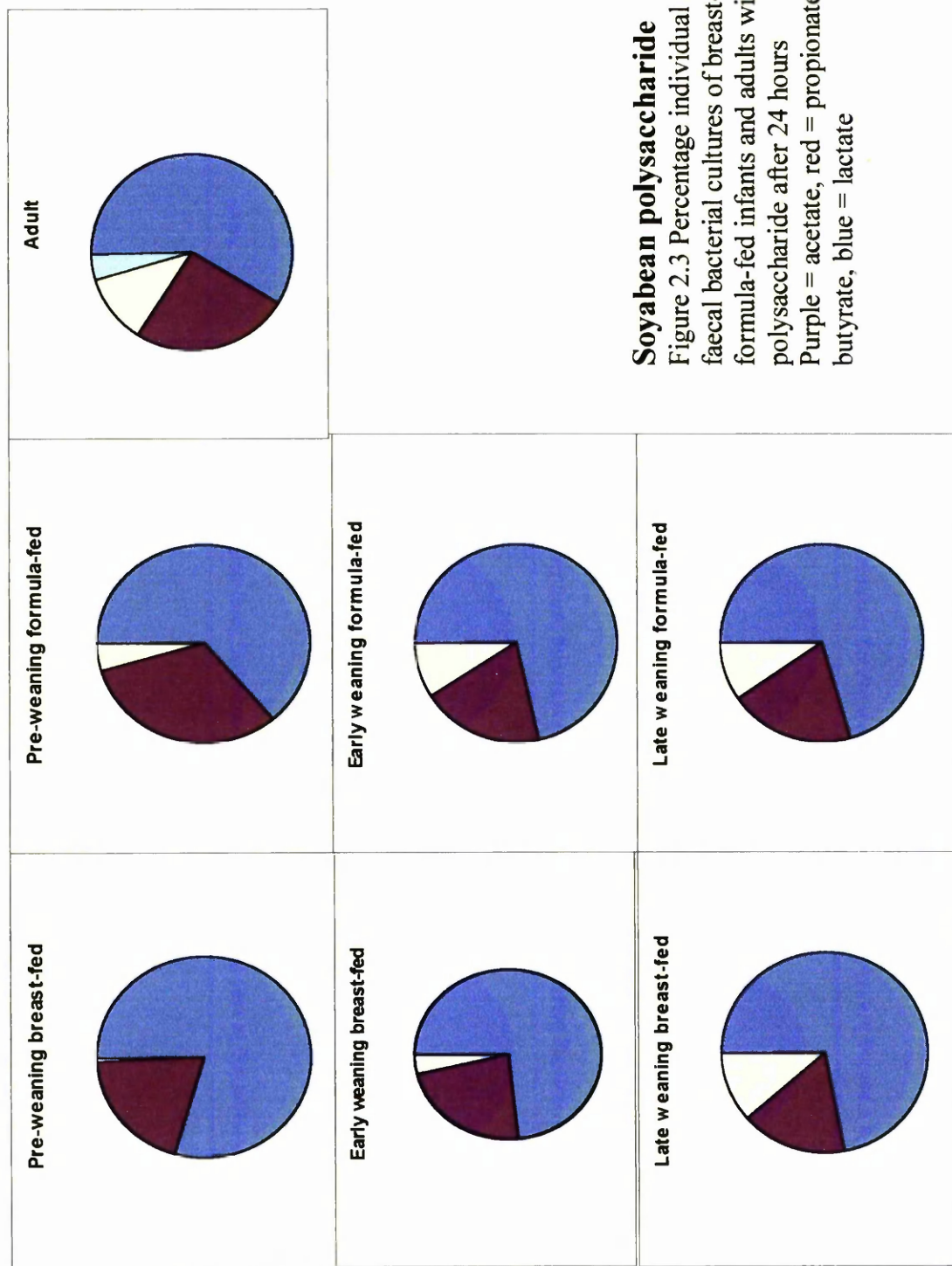
Figure 2.1 Percentage individual SCFA in faecal bacterial cultures of breast-fed infants, formula-fed infants and adults with glucose after 24 hours  
 Purple = acetate, red = propionate, yellow = n-butyrate, blue = lactate



### Raftilose™

Figure 2.2 Percentage individual SCFA in faecal bacterial cultures of breast-fed infants, formula-fed infants and adults with Raftilose™ after 24 hours

Purple = acetate, red = propionate, yellow = n-butyrate, blue = lactate



### Soyabean polysaccharide

Figure 2.3 Percentage individual SCFA in faecal bacterial cultures of breast-fed infants, formula-fed infants and adults with soyabean polysaccharide after 24 hours  
Purple = acetate, red = propionate, yellow = n-butyrate, blue = lactate

**Table 2.3 Median values of individual SCFA after bacterial culture with faeces from breast-fed infants, formula-fed infants and adults with various carbohydrates**

	$\mu\text{mol/ml}$ individual SCFA			
<i>Glucose</i>	Acetate	Propionate	n-Butyrate	Lactate
<b>Breast-fed</b>				
Pre-weaning (n=12)	72.2	0.9 <sup>***†††</sup>	0.2 <sup>**</sup>	25.9
Early weaning (n=7)	65.8	5.1 <sup>+</sup>	1.0 <sup>*</sup>	24.1
Late weaning (n=8)	68.1	11.5 <sup>++</sup>	3.3 <sup>++</sup>	16.7
<b>Formula-fed</b>				
Pre-weaning (n=10)	32.8	9.4	1.3 <sup>*</sup>	7.6
Early weaning (n=10)	51.4	5.6	2.3	10.6
Late weaning (n=10)	49.0	6.2	7.9	5.9
<b>Adult (n=6)</b>	56.7	16.1	6.5	25.1
<i>Raftilose™</i>	Acetate	Propionate	n-Butyrate	Lactate
<b>Breast-fed</b>				
Pre-weaning (n=12)	39.2	4.1	0.4 <sup>*</sup>	5.0 <sup>*</sup>
Early weaning (n=7)	49.2	5.7	3.0	4.6
Late weaning (n=8)	58.7 <sup>++</sup>	14.8 <sup>+</sup>	11.0 <sup>++</sup>	1.2 <sup>*</sup>
<b>Formula-fed</b>				
Pre-weaning (n=10)	34.5	8.0	3.2	2.9 <sup>*</sup>
Early weaning (n=10)	49.3	8.0	7.6	3.2
Late weaning (n=10)	34.9	6.7	4.8	0 <sup>*</sup>
<b>Adult (n=6)</b>	39.1	12.4	11.2	17.6
<i>Soyabean Polysaccharide</i>	Acetate	Propionate	n-Butyrate	Lactate
<b>Breast-fed</b>				
Pre-weaning (n=12)	21.2	6.0 <sup>**</sup>	0.2 <sup>**</sup>	0
Early weaning (n=7)	16.3 <sup>*+</sup>	4.4 <sup>**</sup>	1.1 <sup>*</sup>	0
Late weaning (n=8)	34.8 <sup>†</sup>	8.5 <sup>*</sup>	4.9 <sup>††††</sup>	0
<b>Formula-fed</b>				
Pre-weaning (n=10)	17.7	7.1 <sup>**</sup>	1.3 <sup>*</sup>	0
Early weaning (n=10)	20.1 <sup>*</sup>	5.8 <sup>**</sup>	2.6 <sup>**</sup>	0
Late weaning (n=10)	25.4	5.9 <sup>*</sup>	2.3 <sup>*</sup>	0
<b>Adult (n=6)</b>	34.3	15.1	6.0	2.0

\* p<0.05, \*\*p<0.01, \*\*\* p<0.001 compared with adult † p<0.05, ††† p<0.001 compared with formula-fed infants, + p<0.05, ++ p<0.01, compared with pre-weaning, †† p<0.05 compared with early weaning.

### 2.3.3 Total SCFA in culture supernatant

When results from 24-hour fermentation culture were compared (Table 2.4, Figures 2.4 and 2.5) there was no difference between the total SCFA produced for any of the substrates at any development stage between breast-fed and formula-

fed infants. There were also no significant differences between formula-fed infants and adults. The cultures with soyabean polysaccharide as substrate produced lower total SCFA at each development stage than adults but this did not reach significance. At early weaning in breast-fed infants there was significantly higher total SCFA with glucose as a substrate than for adults ( $p < 0.05$ ).

When comparisons were made between the developmental stages in breast-fed infants, cultures with raftilose™ produced significantly higher amounts of total SCFA at early weaning ( $p < 0.05$ ) and at late weaning ( $p < 0.001$ ). Cultures with soyabean polysaccharide produced significantly more total SCFA at late weaning than at pre-weaning ( $p < 0.01$ ) but significantly less at early weaning than at late weaning ( $p < 0.01$ ). In contrast, there were no significant differences between the formula-fed infants for any substrate at any developmental stage.



**Table 2.4 Short chain fatty acid production in cultures of faecal bacteria from BF and FF infants during weaning.**

		Total SCFA + Lactic acid concentration (μmol/ml)					
		Pre-weaned <i>n</i> = 12		Early weaned <i>n</i> = 7		Late weaned <i>n</i> = 8	
		Median	Range	Median	Range	Median	Range
<b>BREAST-FED</b>							
Glucose		82.3	20.7 -103.0	99.6*	82.0 -120.5	83.4	58.5 - 129.9
Raffilose™		41.3	3.6 - 56.1	62.8†	2.5 - 90.2	76.0††	54.8 - 113.4
Soyabean polysaccharide		11.6	8.1 - 40.2	7.0††	4.4 - 23.9	34.8††	23.2 - 78.4
		Prewedaned <i>n</i> 10		Early weaned <i>n</i> 10		Late weaned <i>n</i> 10	
		Median	Range	Median	Range	Median	Range
<b>FORMULA-FED</b>							
Glucose		56.3	15.8 -107.2	67.7	23.9 -101.3	68.5	10.9 - 155.7
Raffilose™		45.7	17.9 -149.6	61.4	25.0 -111.4	64.5	14.0 - 150.6
Soyabean polysaccharide		14.1	11.5 - 27.2	18.5	3.1 -100.8	26.2	2.2 - 69.4

\*  $P < 0.05$ , †  $P < 0.01$ , ††  $P < 0.001$  compared with pre-weaned infant, †††  $P < 0.01$  compared with late weaned infant.

Figure 2.4 Fermentation capacity of breast-fed infants for glucose, rafterlose™ and soyabean polysaccharide at pre-weaning, early weaning and late weaning

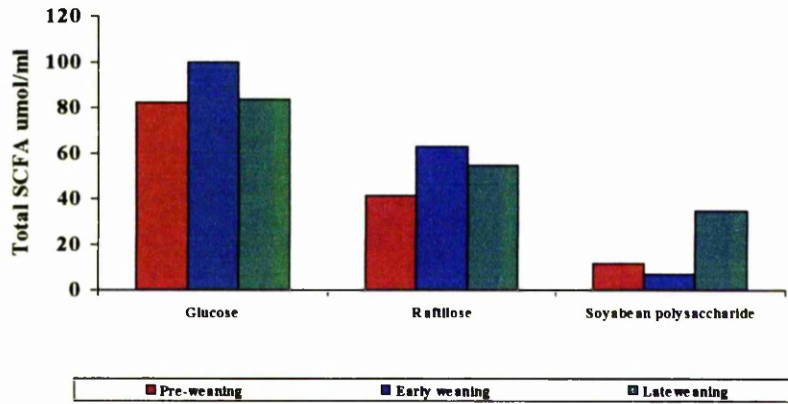
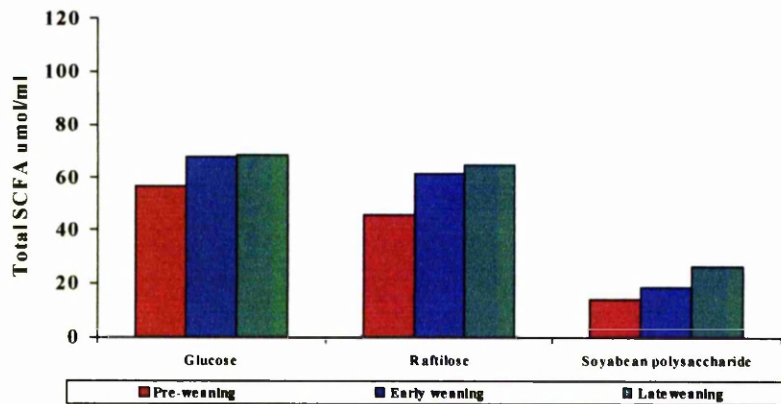


Figure 2.5 Fermentation capacity of formula-fed infants for glucose, rafterlose™ and soyabean polysaccharide at pre-weaning, early weaning and late weaning



## 2.4 DISCUSSION

The production of SCFA depends on the microflora and the unabsorbed food that reaches the colon. Faecal SCFA represent the net sum of production and absorption of SCFA throughout the gastrointestinal tract. In infancy, the microflora is not stable and, as new foods are introduced into the diet, changes may occur. Weaning, when the infant is exposed to new foods, is therefore, thought to be a critical period for the development of the stable adult microflora. In studies in rats (Armstrong *et al.*, 1992) weaning diet was shown to affect later fermentation of dietary fibre. Also studies in mice (Lee and Gemmell, 1972) showed that at the onset of weaning the microflora changed and consequently the profile of SCFA altered.

There have been few studies investigating the microflora and SCFA production during the weaning period. If fermentation capacity is fully developed, the SCFA produced can contribute to the daily energy needs (up to 2kcal/ g) (Livesey, 1990). If the infant is presented with a substrate it is unable to ferment it will pass through the colon unmetabolised, pulling water with it and causing an increase in stool output. This will result in increased faecal energy losses. Prior to weaning, breast-fed infants are less liable to have diarrhoea than formula-fed infants (Howie *et al.*, 1990). However, the weaning period, with the introduction of new substrates, may cause breast-fed infants to be more susceptible to diarrhoea (Gordon, 1971). Formula-fed infants have a microflora that is closer to that of adults prior to weaning so may not undergo so many changes at weaning and consequently at this stage may be less prone to diarrhoea.

In this cross-sectional pilot study, we investigated the ability of breast-fed and formula-fed infants to ferment simple and complex carbohydrates *in vitro* at different development stages. It was shown that there were no differences in fermentation capacity at each development stage when breast-fed infants were compared with formula-fed infants. This may be due to the small number of infants in this study.

However the ability of the breast-fed infants to ferment carbohydrate did increase during weaning especially for raftilose™ and soyabean polysaccharide. Although the breast-fed infants could ferment simple sugars (glucose) readily at all stages of weaning they were less able to ferment raftilose™ (a fructo-oligosaccharide) and soyabean polysaccharide. The ability to ferment raftilose™ increased in early weaning but the ability to ferment soyabean polysaccharide did not increase until late weaning. In contrast, there were no differences between any development stage for any substrate in formula-fed infants. Both infant feeding groups produced less total SCFA with soyabean polysaccharide than adults but this did not achieve statistical significance which may be due to the small numbers studied. This was also reflected in the higher pH in the cultures of infants compared to adults. This indicates the poor ability of infants to ferment complex carbohydrates and suggests that they had not reached the adult capacity for fermenting complex carbohydrates (75% and 57% of total SCFA produced by adults for breast-fed and formula-fed infants respectively) even by late weaning.

It is well established that faecal flora of breast-fed and formula-fed infants differs (Edwards *et al.*, 1994). This may be related to a variety of factors. The neonatal diet (breast or formula milk) may influence the colonisation of the infant gut or there may be a difference in the non-absorbed portion of breast or formula milk which then influences the actions of the bacteria in the gut. Dietary factors which may influence the colonisation of the infant colon include buffering capacity, predominance of whey or casein protein (Balmer *et al.*, 1989), iron or lactoferrin content (Balmer and Wharton, 1989, 1991) and nucleotide content (Gil *et al.*, 1986). Breast-fed infants have acetate and lactate as the predominant SCFA whereas in formula-fed infants have more propionic and n-butyrate and less lactate. In this study, however, the proportions (Figures 2.1, 2.2, and 2.3) show that at pre-weaning with cultures containing glucose and raftilose™ the predominant SCFA are acetate and lactate, but as weaning progresses the proportions of propionate and butyrate increase and lactate decreases. This pattern was different in formula-fed infants acetate and propionate

being predominant at all stages of weaning, although at early weaning there was also a high proportion of lactate. It was not until early weaning that the proportion of butyrate increased. With cultures of soyabean polysaccharide the predominant SCFA are acetate and propionate through all stages of weaning and no lactate is seen at any stage. Lactate is an intermediate electron sink and when produced can be further fermented to allow rapid regeneration of  $\text{NAD}^+$  and fast fermentation. Therefore, the lack of lactate in these cultures is probably due to the much slower fermentation of soyabean polysaccharide. Whilst there was a small proportion of butyrate at pre-weaning, this increased in early and again in late weaning. This pattern was very similar for formula-fed infants.

## 2.5 CONCLUSION

This pilot study indicated differences in the development of fermentation capacity between breast-fed and formula-fed infants. The fermentation capacity of breast-fed infants to ferment complex carbohydrates increased during weaning whereas there were no differences in formula-fed infants. Both groups of infants had a lower fermentation capacity than adults for soyabean polysaccharide although it did not reach statistical significance. This suggests that fermentation capacity is still developing which is further supported by the presence of starch in the faeces of children up to 3 years of age (Verity and Edwards, 1994). Colonic salvage of energy from complex carbohydrates therefore is unlikely to contribute significantly to the energy needs of the infant during early weaning. The greater similarity of the colonic flora of formula-fed infants to that of the adult allows faster adaptation to the complex carbohydrates in a weaning diet than the simpler flora of the breast-fed infant. This may be related to the greater incidence of diarrhoea during weaning of breast-fed infants.

The differences in the fermentation capacity of breast-fed and formula-fed depends on neonatal feeding practice but also may be influenced by foods given at weaning.

This pilot study gave an indication that fermentation capacity develops during weaning but also demonstrated the need for larger numbers of infants and a longitudinal design to draw definitive conclusions. As many infants in Scotland are given a mixture of breast and formula milk it was decided that this group should also be investigated. Standard deviations obtained from the pilot study allowed the calculation of a power equation to determine the sample size needed in the longitudinal study.

*I would like to acknowledge the help of Ella Lokerse for her help in this pilot study.*

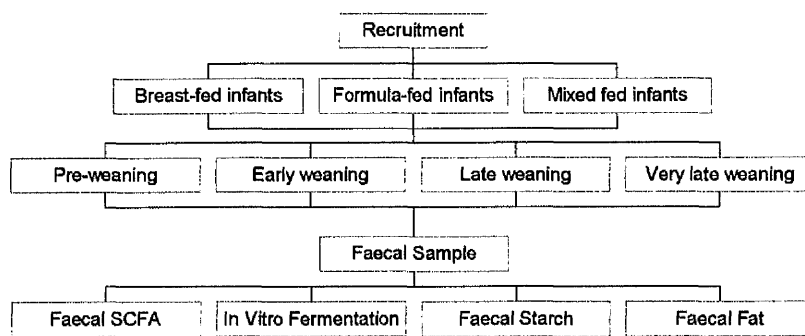
# **Chapter 3**

## **Materials and Methods**

### 3.1 INTRODUCTION

This chapter covers the general methods used in this thesis, more specific methods are given in individual chapters.

### 3.2 STUDY DESIGN



### 3.3 THE SAMPLE SIZE

A power equation (Altman, 1980) utilising data obtained in the pilot study (described in Chapter 2) was used to determine the sample size needed to investigate the difference in concentration of SCFA produced by in vitro fermentation between three groups of infants. The standard deviation for total SCFA in the pilot study was 10  $\mu\text{mol}/\text{ml}$ . In order to detect a difference of 15  $\mu\text{mol}/\text{ml}$ , the power equation concluded that 25 infants in each group were needed at a level of 95% power. It was decided to recruit 50 infants to allow for 50% dropout, which was the dropout rate seen in previous studies.



Recruitment was carried out in The Queen Mother's Hospital, Glasgow and ethical approval was obtained from Yorkhill NHS Trust. Suitable mother/infant pairs were selected from hospital records and invited to take part in the study. Those mothers who agreed to participate gave written, informed consent. Suitability for participation was determined as normal delivery birth (not caesarian), infants recruited had to be full-term and healthy with birth weight over 2250g and subjects had to be within travelling distance to collect the sample quickly (60 minutes return trip).

291 mothers were asked to participate in the study. This involved visiting mothers in hospital shortly after they had given birth and describing the study to them. They were then revisited (24-48 hours later) to ask if they wished to take part. Of the 291 mothers who were approached regarding the study 118 left hospital before a second visit could be made. Of the remainder 46 mothers opted not to take part in the study and 127 mothers agreed to take part. Within the group of mothers that agreed to take part, there were three subgroups; those who participated fully throughout the study (63), those who gave some samples at some developmental stages, but not enough to be followed longitudinally (23) and those who agreed to take part but never gave samples (41). More detail of the infant sample is given in Chapter 4.

### **3.3.1 Collection of samples**

At the appropriate time points - 1 month, 2 months, 4 weeks after beginning weaning, 7 months, 9 months, 1 year, 18 months and 2 years, subjects were asked to give an infant stool sample (in the form of a soiled nappy). Parents would telephone the department when their child had a soiled nappy and this was collected as soon as possible after defaecation (within 1 hour). The sample was brought back to the laboratory for analysis. The faecal material was scraped from the nappy and weighed. Depending on available material, aliquots of faeces were taken for the

various assays. If there was too little faecal sample in the nappy, subjects would be asked to provide up to three fresh nappies in order to carry out the analyses.

Depending on how much faecal material was available, the following were carried out:

- An aliquot of faeces was used to inoculate cultures for an *in vitro* fermentation model. After incubation for 24 hours, pH of the supernatant was measured and SCFA and lactic acid content were measured by gas liquid chromatography (GLC).
- A weighed aliquot was taken, 1 ml of 1M NaOH was added to bring the pH to > 9.0 and the sample was frozen at -20°C. This was used at a later date to assess amounts of SCFA and lactic acid present in the original faecal sample.
- Two further aliquots of faeces were taken; these samples were weighed and then frozen at -20°C. These were analysed later for faecal starch and faecal fat.

### 3.4 MEASUREMENT OF FERMENTATION

For the main studies in this thesis, a good model of colonic fermentation was needed. Before choosing a model the literature was reviewed for potential approaches.

The study of fermentation, the bacteria involved, their metabolic activities and the products formed has a number of methodological problems (Rumney, 1996). The major problem of measuring fermentation *in vivo* is the inaccessibility of the proximal colon where most fermentation is thought to occur (Edwards and Rowland, 1992). Sampling directly from the colon in humans is difficult, and although it is possible to obtain colonic samples from animals, a suitable animal model which reflects human colonic fermentation needs to be chosen. The alternative is to measure products of fermentation excreted in human breath, faeces or urine. To overcome problems associated with *in vivo* systems, a range of *in vitro* systems have

been developed which range in their complexity. A main disadvantage of *in vitro* models is that the inoculum for the systems is usually faeces. Whether faeces is appropriate to use as the inoculum has been questioned and is discussed in more detail in Chapter 1. *In vitro* and *in vivo* methods for studying colonic fermentation in the adult and infant are discussed below in terms of their advantages and limitations and for their suitability for the study described in this thesis.

### **3.4.1 *In vivo* methods**

The methods used for *in vivo* study of colonic fermentation of carbohydrate are expensive, difficult to carry out, and need medical supervision and ethical approval. *In vivo* methods are almost impossible to use for the study of fermentation in infancy.

#### **3.4.1.1 *In vivo* methods (Human)**

Most *in vivo* determination of colonic fermentation is based on indirect measurement of fermentation products; H<sub>2</sub> or SCFA in plasma or faeces.

The only data that has been collected directly from the colon has been from sudden death victims (Cummings *et al.*, 1987). If collected quickly, this may give an accurate picture of the products of fermentation in the colon. Intubation studies are difficult and may interfere with the physiology of the colon. The presence of the intubation tube may alter transit time (Read *et al.*, 1983) or more significantly the wash out necessary before the study would alter the colonic contents. Intubation studies (Florent *et al.*, 1985) have been used successfully, however, to sample from both the ileum and caecum and to follow the appearance and disappearance of fermentation intermediates. These are very specialised techniques however, and cannot be used routinely.

Ileostomy patients have been used to gain information on nutrients that escape digestion and absorption in the small bowel. Patients with stomas would enable access to the colonic lumen and a direct measure of colonic function. However, colonic flora of these patients may not be comparable to healthy adults and therefore may be of limited use (Mitchell *et al.*, 1985; Finegold *et al.*, 1970; Segal and Hassan, 2000).

Other methods of sampling colonic contents include ingestion of a dialysis bag or radiotelemetric pills (Wrong *et al.*, 1965). After ingestion, the contents of the gut equilibrate with the contents of the dialysis bag and SCFA can be measured after egestion from the body. Radiotelemetric pills measure luminal pH as they pass down the gut (Evans *et al.*, 1988). This allows study of pH in different parts of the gut after ingestion of a polysaccharide. However, fermentation of some resistant polysaccharides does not decrease colonic pH very much. None of these approaches are suitable in infancy.

#### **3.4.1.1.1 Breath hydrogen**

The breath hydrogen (H<sub>2</sub>) test is the most commonly used indirect test for measuring carbohydrate fermentation (Rusmessen, 1992). H<sub>2</sub> breath tests are non-invasive and non-radioactive, and they do not disturb normal physiological functions. The basis of this test is that all the H<sub>2</sub> produced in man results from fermentation by the colonic bacterial flora. A proportion of the H<sub>2</sub> produced is absorbed through the mucosa into the blood and then excreted in breath (Levitt, 1969).

When the food reaches the colon, hydrogen rises as fermentation of unabsorbed carbohydrate begins. This first sustained rise in breath hydrogen concentration corresponds to the mouth to caecum transit time (Bond and Levitt, 1975). The rise in breath H<sub>2</sub> concentrations after MCTT (mouth to caecum transit time) can be quantified by the maximal rise from baseline or more accurately by the area under the

curve (AUC) in a sufficient period of time (Rusmessen, 1992) for the hydrogen to return to baseline or at least approached baseline values.

However, breath hydrogen measurement values are not a good quantitative measure of carbohydrate malabsorption because there are several other routes for hydrogen disposal, methane, H<sub>2</sub>S and acetate production (Gibson *et al.*, 1990). There also may be a wide variation in baseline depending on background diet and antibiotic use (Gilat *et al.*, 1978). It is essential for a useable breath test for the subject to fast overnight for 8-12 hours to produce a very low and steady background breath hydrogen below 5pm (Rusmessen, 1992), indeed fibre containing foods may need to be avoided for the whole of the previous day.

Florent *et al.*, (1985) showed that ingestion of lactulose for a week reduced the amount of hydrogen produced although fermentation efficiency actually increased. It is also important to choose an appropriate control carbohydrate. Lactulose, which is most often used, is not a good model for slowly fermented carbohydrates such as resistant starch.

#### **3.4.1.1.2 Plasma SCFA**

Plasma acetate, measured in peripheral blood, is an alternative method for assessing fermentation. Acetate is the major SCFA produced from fermentation of carbohydrate (Cummings and Englyst, 1987) and is the only SCFA that remains in the blood after passage through the liver. Acetate has been used to monitor the fermentation of dietary fibre (Pomare *et al.*, 1985) and a few studies have noted a general increase in blood acetate after resistant starch fermentation (Muir *et al.*, 1995). However, it is difficult to quantify fermentation using plasma acetate alone. Many blood samples are needed over each study day and this presents practical and ethical difficulties. Also separation of endogenous acetate from acetate produced

during fermentation can be difficult if the carbohydrate is slowly fermented and a long period is required to follow the rise and fall in acetate. This could be helped by ingestion of small meals throughout the day but the food given may also affect the fermentation process.

Stable isotope labelled acetate offers an advantage in separating endogenous production from fermentation. It can be utilised either directly by measuring labelled acetate produced by fermentation of  $^{13}\text{C}$  labelled carbohydrate or indirectly by loading the blood with  $^{13}\text{C}$  acetate and measuring dilution by fermentation products (Pouteau *et al.*, 1998). Techniques using blood samples would not be suitable for studies in infants.

#### **3.4.1.1.3 Faecal SCFA**

Many studies measure faecal constituents which, although collection may be unpleasant, can be obtained with relative ease. Fermentation will continue after defaecation so it is important to process samples as soon as possible after passage. Faecal SCFA will change little if a rapidly fermentable carbohydrate is eaten, as almost of the SCFA produced will be absorbed before the faeces is produced. A rise in SCFA, or a change in the profile of SCFA, may indicate prolonged fermentation especially in the distal colon and the amount of the carbohydrate left in the stool will provide evidence of fermentability (Nyman and Asp, 1988). However, quantitative data of SCFA production are not possible from faeces. This emphasises the need for animal models, which allow measurement of caecal and colonic SCFA.

Other non routine methods for collecting human colonic contents have included material collected post mortem after sudden death for direct analysis (Stephen *et al.*, 1987), or for supply of inoculum for *in vitro* studies (Macfarlane *et al.*, 1992). More invasive techniques *in vivo* that have been used are needle aspiration from patients

during surgery, (Bentley *et al.*, 1972) and the swallowing of capsules that allow the remote control sampling of gastrointestinal tract contents (Pochart *et al.*, 1993). Obviously, these methods of sample collection are not available for most studies but have provided important insights into colonic fermentation processes.

#### **3.4.1.1.4 Stable isotopes breath tests**

A new approach to fermentation measurements may lie in the use of stable isotope labelled carbohydrates. The stable isotope substrates may be obtained from naturally enriched C4 plants such as corn and cane sugar but other carbohydrates need to be labelled artificially, by the source plant being grown in  $^{13}\text{CO}_2$  (Edwards *et al.*, 1998).

Ingestion of  $^{13}\text{C}$  labelled fibre or starch will enrich the level of  $^{13}\text{CO}_2$  in the breath (Schoeller *et al.*, 1980) if adequate enrichment of  $^{13}\text{C}$  label in the food is achieved. This  $^{13}\text{CO}_2$  will arise from small intestinal digestion and absorption of starch, followed by cellular metabolism of glucose, or directly from bacterial fermentation of carbohydrate and also cellular metabolism of SCFA. The small intestinal and colonic events are separated by time producing more than one peak in  $^{13}\text{C}$  enrichment in the breath (Edwards *et al.*, 1998, Vonk *et al.*, 1998). The resulting curves need mathematical models to separate the different small intestinal and colonic fermentation components (Morrison *et al.*, 1998). This is still an invalidated method but it is non-invasive and safe and could be used in infants, children as well as adults (Christian *et al.*, 1998).

### **3.4.1.2 *In vivo* Methods (Animal)**

#### **3.4.1.2.1 The conventional animal model**

Animal models allow controlled dietary conditions, long term studies and access to the contents of the caecum, proximal and distal colon. They also allow study of the interaction between the fermentation process and the gut wall. There is, however, little standardisation of conditions for animal feeding trials. Variations in basal diets (basal diets containing from zero to 16g of fibre per day (Edwards *et al.*, 1992a); length of study period (less than 1 week-18 months [Edwards *et al.*, 1992a]), and level of fibre inclusion (increased over basal diet by 1.5g [Edwards *et al.*, 1992b] - 10g per day [Nyman and Asp, 1988]) all contribute to large differences in results seen between studies. A fibre containing basal diet may mask the effects of the carbohydrate being studied. Colonic bacteria may take over 4 weeks to adapt to a new carbohydrate (Walter *et al.*, 1986).

Choosing a suitable animal model is also a problem. The most frequently used animals are rats and pigs. The rat has a large caecum and a shorter transit time than man (Van Soest *et al.*, 1982). The rat is a caecal fermenter and coprophagy is very difficult to control. The rat colonic microflora is similar but not identical to that of man (Donaldson, 1968), and rats produce relatively more butyric acid on high fibre diets (Topping *et al.*, 1985). The effects of dietary fibre in the rat, however, are well correlated with their effects in man (Edwards *et al.*, 1992a).

The pig is a useful animal model also with similar microbial populations to man (Donaldson, 1968) but is expensive and needs specialist facilities. The pig has a larger caecum and greater capacity for fibre fermentation than man but has similar blood and faecal short SCFA (Graham and Aman, 1987).

There is, at present, no conventional animal model of the infant gut.



#### **3.4.1.2.2 Gnotobiotic animal models**

Where facilities for germ-free animals are available, these can be used as an excellent control for the impact of fermentation on the animal. Germ free rats can also be associated with human colonic microflora to provide a model of the colonic microflora that is more comparable to the human. A useful model is the germ free rat colonised with bacteria from a human faecal sample. It has previously been demonstrated that rats, born germ-free, maintained in flexible-film isolators and associated with a human microflora by orally dosing with a fresh human faecal suspension retain the bacteriological (Hazenberg *et al.*, 1981) and metabolic (Mallett *et al.*, 1987; Hirayama *et al.*, 1995) characteristics of the human flora. In addition, such animals, termed ‘human-flora-associated’ (HFA) rats, respond to a dietary change in a manner characteristic of the human rather than the rat microflora.

Recently a HFA model of the breast-fed infant gut has been described (Edwards *et al.*, In press) and could be used for studying weaning foods. However, the expense of these studies and the limited number of available facilities reduces the potential use of this model.

#### **3.4.2 *In vitro* models**

For initial evaluation of fermentability of foods, food ingredients, potential prebiotics and nutraceutical products (foods used for therapeutic purposes), *in vivo* methods are not suitable. Simple, quick and cheap methods are needed. Several *in vitro* methods have been described for the estimation of colonic fermentation *in vitro*. There are two main approaches, the batch or static culture and the continuous culture method.

### 3.4.2.1 Static (batch) cultures

There have been two major ring trials of fermentation methods (Barry *et al.*, 1995 and Edwards *et al.*, 1996). Barry co-ordinated a major trial of a standardised method (Table 2) used to study fermentation of dietary fibre examining the importance of inter- and intra-individual variability, and inter-laboratory variation. This was a simple model using an inoculum consisting of fresh human faeces mixed with a carbonate – phosphate buffer supplemented with trace elements and urea. The trial found that the fibres tested were ranked in similar order by all laboratories despite some differences in absolute values for SCFA production and fibre degradation.

A smaller ring trial tested a standardised method for assessing the fermentation of resistant starch (Edwards *et al.*, 1996). The model was simpler than that of Barry *et al.*, using buffer only without trace elements or urea (Table 3.1) but showed good agreement between laboratories.

<b>Table 3.1                      Standardised and tested <i>in vitro</i> models of fermentation</b>		
	<b>Barry <i>et al.</i>, 1995</b>	<b>Edwards <i>et al.</i>, 1996</b>
Medium	Carbonate buffer Macrominerals Trace elements Urea	Phosphate buffer pH 7.0
Inoculum	20 g/litre	16g/litre
Substrate	10g/l	10g/l
Shaking	Shaking water bath/ orbital shaker	Shaking water bath/ orbital shaker 50 strokes/min
Duration	0, 6, 10 – 24 hours	0, 2, 4, 24 hours
End points	Residual NSP pH SCFA	Residual Starch pH SCFA and lactate

*In vitro* models of the infant colon are more limited in number. Infant studies have particular problems. The small faecal output of infants, especially pre-weaned breast-fed infants (0.5-5g), means that models have to be adapted and scaled down. The

assay involves collecting and processing the faecal sample within one hour of defaecation and there are problems associated with obtaining samples at appropriate times from infants. There is a major difference in the predominant flora dependent on method of feeding. Breast fed infants have a flora consisting mainly of bifidobacteria and lactobacilli which are less easy to maintain in mixed cultures *in vitro* (Edwards *et al.*, 1985). Table 3.2 gives differences between adult and infant models.

<b>Table 3.2 Variations in methods for <i>in vitro</i> fermentation model.</b>		
<b>Model Variable</b>	<b>Adult models</b>	<b>Infant models</b>
Faeces concentration	0.5-250 g/l	0.032 g/l
Substrate amount	10-50 g/l	105 µmol/g stool homogenate
Buffer	none, phosphate buffer or carbonate phosphate buffer, mostly pH 6.5 – 7.5	0.1 M phosphate buffer pH 7.0
Added amino acids	none, yeast extract or trypticase	none
Minerals	none, macrominerals, trace elements	macrominerals, microminerals
Mixing or shaking	none, shaking water bath or periodic shaking	none
Duration	1-168 hours most cultures 24-48 hours	1 hour, 24 hour
References	Barry <i>et al.</i> , 1995 Edwards <i>et al.</i> , 1996	Lifschitz <i>et al.</i> , 1990 Parrett & Edwards, 1997

The limitations of the batch culture method include an initial lag phase, product inhibition, which may distort fermentation results especially if a low pH is produced, and a limited life of not much more than 24 hours. The cultures must also be maintained under strict anaerobic conditions. Some of these problems are overcome in continuous culture models.

### 3.4.2.2 Continuous culture models

The continuous and semi-continuous culture models aim to mimic the human colon more closely and to maintain the bacterial flora in steady state and so have many advantages over the static cultures. However, they are more expensive and more labour intensive. Several different approaches have been taken. The medium may be replenished continuously (Veilleux and Rowland, 1981) or at intervals during the day to mimic the periodic entry of ileal chyme into the colon (Miller and Wolin, 1981). The models vary in complexity from those using one vessel (Miller and Wolin, 1981; Edwards *et al.*, 1985) to models in which two or more vessels are placed in series to provide more heterogeneity of physico-chemical conditions and to mimic pH gradients in the colon (Veilleux and Rowland, 1981; Gibson *et al.*, 1988). On the basis of the production of SCFA, these models appear to mimic bacterial fermentation in the colon quite closely. However, there is still a lack of rapid removal of SCFA from the system apart from that which results from daily dilution with fresh medium.

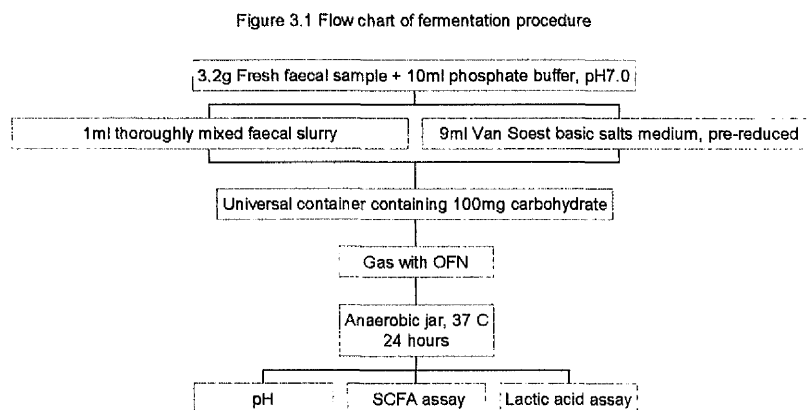
A dynamic model has been developed by researchers at TNO Nutrition and Food Research at Zeist in The Netherlands (Minekus *et al.*, 1995). This model is composed of all the major constituents of the gastrointestinal system and simulates peristaltic movement, physiological pH curves, and absorption of water and nutrients, physiological emptying patterns and adjustable gastric and intestinal transit times. The whole system is controlled and monitored by computer. Although useful the system is prohibitively expensive to most laboratories and still has no epithelium or way of mimicking the immune system.

A continuous culture model of the infant gut has recently been developed (Parrett *et al.*, In press) but this was developed after the current study was concluded and also would not be of use in studying the fermentation properties of different individual infants.

Given the aim of the present study to determine the differences between individual infants a static *in vitro* model was chosen. This *in vitro* method was chosen as it was relatively simple, rapid and cheap. It allowed a large number of samples to be fermented at once. In addition it was a method that enabled the use of smaller faecal samples which were likely to be obtained from infants. The media contained all the potential growth requirements of this simplified flora which were not included in the methods of Barry *et al.*, and Edwards *et al.*. In those models the faeces was assumed to provide these but this may not be the case in the much smaller infant samples.

### 3.5 *IN VITRO* FERMENTATION IN THIS STUDY

Fermentation capacity was tested in a *in vitro* model modified from that of Adiotomre *et al.*, 1990. An overview of the *in vitro* fermentation method is given in Figure 3.1.



Faeces were scraped from the soiled nappy and weighed within 1 hour of defaecation to ensure the system was mimicking the bacteria of the gut as closely as possible by prevention of loss of anaerobic species. An aliquot of the faecal sample was used to

set up an in vitro fermentation system. The aliquot was weighed to make a 32% faecal slurry with Sorensens phosphate buffer, pH 7.0. In most cases 3.2g of fresh faeces was used from the sample, unless only a small amount of faecal material was available in which case the amounts of all reagents and culture volume were scaled down (usually by half). The faeces were mixed using a vortex mixer for 2-3 minutes with 10 ml of pre-reduced Sorensens phosphate buffer (pH 7.0). This was to give a homogeneous faecal slurry (32% w/v). One ml of faecal slurry was used to inoculate 9 mls of pre-reduced basic salts solution (Van Soest solution; Adiotomre *et al.*, 1990), containing 100 mg of carbohydrate substrate (Table 3.3).

<b>Table 3.3 Description and source of substrates used</b>		
<b>Substrate</b>	<b>Description</b>	<b>Source</b>
Glucose	Easily fermented by all bacteria	Merck, Loughborough, UK
Lactose	Main carbohydrate source for infants	Sigma Chemical Company, Poole, UK
Raftilose™	Small quantities present in breast milk	Raffinerie tirlémontoise, SA
Soyabean polysaccharide	Often used in weaning foods	Scientific Hospital Supplies UK Ltd., Liverpool, UK
Pectin	Present in fruit which is often given at weaning	Merck, Loughborough, UK
Raw potato starch	Used as a model of resistant starch which may be produced in the processing of weaning foods	Rochet, France

### 3.5.1 Carbohydrate Substrates

Substrates were chosen to reflect a range of simple and complex carbohydrates (Table 3.3). Glucose is readily fermented by most bacteria and allows comparison of potential fermentation capacity more readily than do sugars or carbohydrates that need induction of enzymes. It is a major component sugar of many dietary fibres, resistant starch and maltodextrins. Lactose is the main carbohydrate present in

human milk. Raftilose™ is chosen as an example of a fructo-oligosaccharide, which may be present in significant quantities in breast milk. Soyabean polysaccharide (in soy flour) is used in weaning foods and is likely to reflect the sort of polysaccharides present in early and late weaning. Pectin and resistant starch were also included to study the relative fermentation capacity for different substrates in more detail.

### 3.5.2 Culture conditions

The cultures were mixed thoroughly, using orange sticks if necessary, the lids of the universal containers were loosened and the contents gassed with oxygen free nitrogen before they were placed in an anaerobic jar. An anaerobic gas pack (Fisons, UK) was added to the jar, the gas pack was opened and water was added, when this was in the presence of a palladium catalyst (Fisons, UK), CO<sub>2</sub> and H<sub>2</sub> were generated creating an anaerobic atmosphere. The jar was incubated at 37 °C for 24 hours. A control culture containing no carbohydrate substrate was set up and incubated in the same way.

After 24 hours the universals were removed from the anaerobic jar and the pH of the fermentation fluid was measured (Kent pH meter, model EIL 7045/7046) after calibration of the pH meter. Universals were then stored at -20 °C for later analysis of SCFA and lactic acid.

#### 3.5.2.1 Solutions used for fermentation process

##### Sorensens phosphate buffer

Solution A	1/15 M KH <sub>2</sub> PO <sub>4</sub>	31 ml	Mixed, boiled and cooled with oxygen free nitrogen (BOC). Made fresh before use.
Solution B	1/15 M Na <sub>2</sub> HPO <sub>4</sub> ·2H <sub>2</sub> O	69 ml	

##### Van Soest Solution

Tryptone	0.5g	To each 40 ml of Van Soest solution 2 ml of reducing solution was added. The solution was mixed, boiled and then cooled with oxygen free nitrogen. The pH of the solution was adjusted to pH 7.0 by using 10% HCL. This was made fresh before use.
Micromineral solution	25µl	
Buffer solution	50 ml	
Macromineral solution	50 ml	
Resazurin (1% resazurin solution)	0.25 ml	

**Buffer Solution**

Distilled water	1L
Ammonium bicarbonate	4g
Sodium bicarbonate	35g

**Micromineral Solution**

CaCl <sub>2</sub> .2H <sub>2</sub> O	13.2g	Distilled water was added to make up to 100 ml.
MnCl <sub>2</sub> .4H <sub>2</sub> O	10.0g	
CoCl <sub>2</sub> .6H <sub>2</sub> O	1.0g	
FeCl <sub>3</sub> .6H <sub>2</sub> O	8.0g	

**Macromineral Solution**

Distilled water	1L
Na <sub>2</sub> HPO <sub>4</sub> .2H <sub>2</sub> O	8.9g
KH <sub>2</sub> PO <sub>4</sub> , anhydrous	6.2g
MgSO <sub>4</sub> , anhydrous	0.36g

**Reducing Solution**

Cysteine hydrochloride	625 mg
Sodium sulphide.9H <sub>2</sub> O	625 mg
Distilled water	95 ml
Sodium hydroxide (1M)	4 ml

The buffer, micromineral and macromineral solutions were made up prior to use and stored in the fridge. The reducing solution had to be made up fresh on the day of use.

### 3.6 SHORT CHAIN FATTY ACID ANALYSIS

#### 3.6.1 Basic principle

SCFA concentrations were measured in both the culture samples and dried faecal samples. Acidified ether extracts were collected to measure SCFA by GLC analysis (Spiller *et al.*, 1980).

A mixture of acetic acid, propionic acid, iso-butyric acid, n-butyric acid, iso-valeric acid and n-valeric acid were used to make an external stock solution (see section 3.6.3). This external standard mixture was used to plot a calibration curve that allowed calculation of each of the SCFA in the test sample.



A series of external standard volumes (10 $\mu$ l, 25 $\mu$ l, 50  $\mu$ l, 100  $\mu$ l, 200  $\mu$ l and 300  $\mu$ l) were analysed to obtain the calibration curve. Distilled water was added to give a final volume of 800 $\mu$ l. 100 $\mu$ l of internal standard -  $\beta$ -methyl valeric acid (Sigma Chemical Company Ltd., Poole, UK) and 100 $\mu$ l of orthophosphoric acid (Merck, Loughborough, UK) was added.

Liquid test samples (800 $\mu$ l) from the fermentation fluid were treated similarly but without the addition of external standard and distilled water. For freeze-dried samples an exact known weight of approximately 100 mg was used with 800 $\mu$ l of distilled water but again no external standard.

Tubes were vortexed to ensure thorough mixing. Three ml of diethyl ether, Analar (Merck, Loughborough, UK) was added before vortexing for one minute. The ether layer, which was at the top of the tube, was removed by pastette and placed in a clean tube. This extraction process was repeated twice more and the extracted ether layers were pooled.

### **3.6.2 Internal Standard**

1g (1.075 ml) of  $\beta$ -methyl valeric acid (Sigma Chemical Company Ltd., Poole, UK) was made up to 100 ml with distilled water. This was brought to pH 7.0 with 0.34g NaOH (Merck, Loughborough, UK).

### **3.6.3 External Standard**

A stock external standard solution was made up of a mixture of SCFA. This external standard was used in the SCFA assay at various volumes to produce a calibration curve from which SCFA in test samples could be calculated.

**Table 3.4 External Standard Stock Solution**

SCFA	Volume	Concentration
Acetic acid	0.95 ml	0.167M
Propionic acid	1.01 ml	0.135M
Isobutyric acid	1.05 ml	0.114M
n-Butyric acid	1.04 ml	0.114M
Isovaleric acid	1.08 ml	0.111M
n-Valeric acid	1.06 ml	0.111M

These were placed in a volumetric flask with 2.9g NaOH (Merck, Loughborough, UK) to adjust to pH 7.0 before making up to 100 ml with distilled water.

All chemicals from Sigma Chemical Company Ltd., Loughborough, UK.

### 3.6.4 GLC conditions

Short chain fatty acids were measured using a Pye Unicam 304 series GLC, this was connected to a Hitachi model 3006 chart recorder. The column (Phase Separations, Deeside, Wales) was a glass column 4 feet in length, the external diameter was 1/4" and the internal diameter was 4 mm. The packing material was 10% SP1000 + H<sub>3</sub>PO<sub>4</sub> on Chromosorb WAW 80-100 mesh. The detector was a flame ionisation detector.

Conditions were as follows:

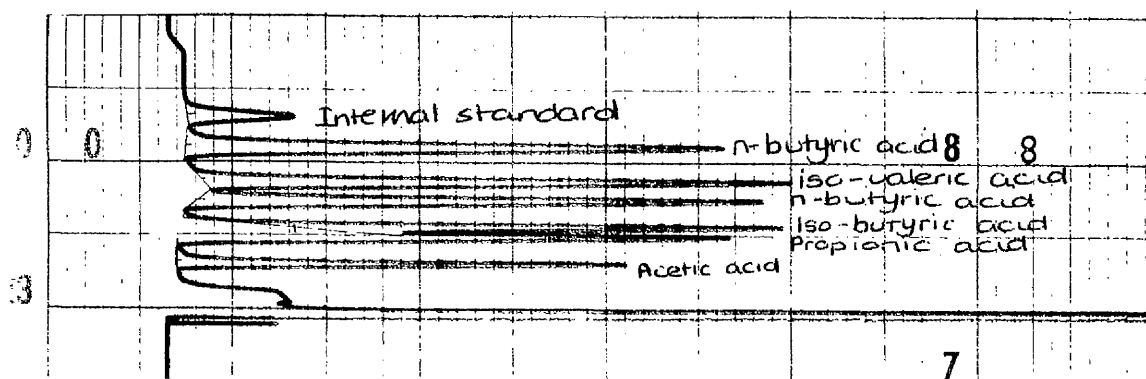
<b>Gas flow rates</b>	carrier gas	nitrogen
	flame gases	air hydrogen
<b>Temperature</b>	Column	200°C
	Injector	180°C
	Detector	200°C
<b>Running conditions</b>	lower temperature	125°C
	upper temperature	150°C
	start hold time	2 minutes
	end hold time	1 minute
	Rate	10°C per minute

Both samples and standards were injected as 3µl onto the GLC column using an SGE 10µl syringe (Hamilton Syringes, UK). Between injections the syringe was washed out with diethyl ether to prevent contamination and carry over. A complete run under these conditions was 10 minutes.

Samples produced from fermentation fluid by acidified ether extraction were run on the GLC and produced peaks on the chart recorder. In the standards the peaks appeared in the following order; acetic acid, propionic acid, iso-butyric acid, n-butyric acid, iso-valeric acid, n-valeric acid and the internal standard (see Figure 3.3). In the samples the peaks produced depended on SCFA present in the sample, and the internal standard peak was always present. Retention times of peaks in test samples were measured against those in the standard to identify individual SCFA.

Peak heights on the chromatogram were measured, a ratio was obtained for SCFA peak height/internal standard peak height. This enabled the quantitation of volumes of individual SCFA.

Figure 3.3 Example of standard run on GLC



### **3.7 LACTIC ACID ANALYSIS**

Lactate concentrations were measured in both the culture samples and dried faecal samples. Methylated ether extracts were measured for lactic acid by GLC (Holdeman and Moore, 1973).

A mixture of lactic acid and phenyl acetic acid were used to make an external stock solution (see section 3.7.2). This external standard mixture was used to plot a calibration curve that allowed calculation of lactate in the test sample.

A series of external standard volumes (25µl, 50 µl and 100 µl) were analysed to obtain a calibration curve. Distilled water was added to give a final volume of 500µl. 200µl of 50% H<sub>2</sub>SO<sub>4</sub> (concentrated sulphuric acid, Merck, Loughborough, UK) and 1 ml of methanol, Analar (Merck, Loughborough, UK) were added. The tubes were stoppered and incubated in a water bath at 55°C for 30 minutes.

Liquid samples from fermentation fluid (500µl) were treated similarly without the addition of external standard and distilled water. For freeze dried samples a known amount of approximately 100 mg was weighed in to tubes and 500µl of distilled water was added but again without the addition of external standard.

After incubation 100µl of orthophosphoric acid was added and the tubes were vortexed to ensure thorough mixing. Ether extraction was with three times 1.5 ml of diethyl ether. The extracts were pooled in a clean tube and this sample was used on the GLC.

#### **3.7.1 Internal standard**

1g of succinic acid dissolved in 100 ml distilled water.

### 3.5.2 External standard

1g (833 $\mu$ l) of lactic acid and 1g of phenylacetic acid were dissolved in 100 ml of distilled water.

### 3.7.3 GLC conditions

Lactic acid was measured on a Pye Unicam 4550 series GLC attached to a PM8251 chart recorder. The column (Phase Separations, Deeside, Wales) was a glass column 4 feet in length, 1/4" outer diameter and 4 mm internal diameter. The packing was 10% SP1000 + 1% H<sub>3</sub>PO<sub>4</sub> on Chromosorb WAW 80-100 mesh. Conditions were as follows:

<b>Gas flow rates</b>	carrier gas	helium
	flame gases	air
<b>Temperature</b>		hydrogen
	Column	200°C
	Injector	180°C
<b>Running conditions</b>	Detector	200°C
	lower temperature	117°C
	upper temperature	146°C
	start hold time	1 minute
	end hold time	0 minute
	Rate	5°C per minute

For both standards and samples 3 $\mu$ l was injected onto the column using a 10 $\mu$ l syringe (Hamilton Syringes) and between injections the syringe was washed through with diethyl ether (Merck, Loughborough, UK) to prevent contamination and carry over.

Narrow peaks were recorded by the chart recorder after each injection. When standards were injected three peaks were produced, lactic acid, succinic acid and phenyl acetic acid. When samples were injected presence or absence of the lactic

acid peak was determined. Peak heights on the chromatogram were measured, a ratio was obtained for lactate peak height/internal standard peak height. This enabled the concentration of lactate to be calculated.

#### **3.7.4 Choice of internal and external standards for lactate analysis**

A range of acids, lactic, phenylacetic, fumaric, succinic, oxalacetic, methylmalonic and pyruvic, were made up as single solutions (1g/ 100mls distilled water) and also a mixture of all these acids were made up as one solution (all at 1g/ 100ml). These solutions were then used as external standards in the lactic acid assay and run on the GLC as described above. The retention times of each acid allowed a choice to be made as to which was the best acids to use as external standard along with lactate and which to use as an internal standard.

Figure 3.4 is the chromatogram produced from the mixture of acids. Lactic acid has a retention time of 75mm, succinate (retention time 205 mm) and phenylacetate (320 mm) were chosen as internal and external respectively. These acids were chosen as they were sufficiently different retention times so as not to get confused with the lactic acid peak. Phenylacetate was added to the lactate external standard as it produces a peak towards the end of the run, whereas succinate produces a peak midway between lactate and phenylacetate. There was no succinic acid present in the infant samples to effect its use as an internal standard.

### **3.8 REPRODUCIBILITY**

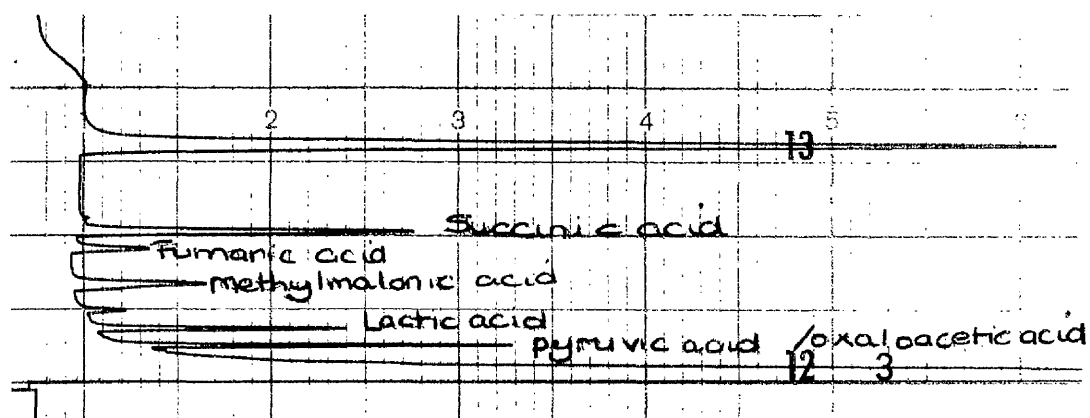
In order to estimate the reproducibility in the SCFA and lactic acid assays a faecal sample was tested several times. The sample was made up incubating an infant faecal sample with glucose using the in vitro fermentation method described earlier in this Chapter. The sample was assayed for SCFA and lactic acid 14 times and the

amounts of each individual SCFA calculated. The % error was calculated as  $[(\text{standard deviation} / \text{mean}) \times 100]$ .

**Table 3.5 Reproducibility of analysis of SCFA and Lactate**

	Acetate	Propionate	Butyrate	Lactate
Mean	13.90	9.44	0.74	12.54
Standard deviation	0.37	0.60	0.03	0.32
<b>Reproducibility</b>	<b>2.69</b>	<b>6.32</b>	<b>4.17</b>	<b>2.52</b>

Figure 3.4 Chromatogram of mixture of acids



### 3.9 ANALYSIS OF FREEZE DRIED FAECES

The sample aliquot taken for faecal SCFA and lactate analysis was weighed, then frozen at -20 °C. It was then freeze dried (Edwards Micromodulyo 1.5K Freeze Dryer, Edwards High Vacuum International, England) and the dried weight was measured. The dried sample was then stored for later analysis of SCFA and lactic acid by GLC. The wet and dry weight of the faecal sample were used to determine water content of the faeces.

$$\% \text{ water} = 100 - [(\text{wet weight} - \text{dry weight}) / \text{wet weight}]$$

Freeze dried samples were analysed for SCFA and lactate as described in sections 3.6 and 3.7.

### 3.10 DETERMINATION OF FAECAL FAT

Fat is a major source of energy to the infant contributing 40-50% of the calories in human milk and most infant formulas (Harries, 1982). New-born breast-fed infants consume 5-7.5 g fat/ kg body weight/ day, which is the equivalent of 350-525g/ day for an adult (Harries, 1982).

Pancreatic lipase and esterase activity are low in newborns and this imposes a constraint on the ability of the infant to hydrolyse and solubilise fat (Harries, 1982). Balance studies have shown there is a 'physiological' malabsorption of fat in newborn infants (Rey *et al.*, 1982) but as gastrointestinal functions mature fat absorption increases. In breast-fed infants, however, lipid absorption is more efficient. This is due to the different composition of the triglycerides found in human milk as opposed to cow's milk. Also in breast milk there is a bile salt activated lipase present which allows digestion of fat droplets of human milk in the duodenum (Guandalini, 1991).



Although even a newborn infant can absorb 60-75% of ingested fat a substantial amount is not digested and reaches the colon (Ling and Weaver, 1997). The fate of the fat in the colon has not been well investigated. It has been proposed that it may have an important influence on the microflora (Thompson and Spiller, 1996) and function of the colon (Spiller *et al.*, 1986). Other studies have suggested it may have a role in the aetiology of colonic cancer (Bull *et al.*, 1984). If fat is not metabolised in the colon it will pass through to the faeces. Most faecal fat is of dietary origin (Schmitz and McNeish, 1987). This excretion of fat in the faeces may therefore contribute to faecal energy losses.

A rapid method for the determination of faecal fat was described by Van de Kamer *et al* in 1949. This method usually relies on a full 5 day stool collection in order to take into account variations in excretion however this was not possible under the conditions of the experimental work undertaken. The Van de Kamer method was therefore modified to use a one off faecal sample to obtain a “snapshot” of fat malabsorption in infants.

### **3.10.1 Principle of faecal fat method**

Neutral fats (glycerides) are saponified with alkali to yield glycerol and soaps; fatty acids are liberated from the soaps with acid extracted and titrated against standard alkali.

### **3.10.2 Subjects**

A sub-sample of the experimental population was used to investigate fat malabsorption in infants. Ten exclusively breast-fed infants were compared with 10 exclusively formula-fed infants. Faecal samples were collected from these infants at pre-weaning (1 - 2 months), early weaning (four weeks after giving solid food) and late weaning (9 months – 1 year). Samples were collected within one hour of

defaecation and scraped from the nappy into a universal container. This was then stored at -20°C for later analysis of faecal fat.

### **3.10.3 Faecal fat analysis**

A single aliquot of faeces of known weight was collected from these infants at each development stage. Faecal fat analysis was carried out in batches so the samples were stored at -20°C until required for the assay. The faecal samples were then allowed to thaw completely before use.

Standard alcoholic sodium hydroxide was used in the titration of the faecal fat determination. 4.0g NaOH was dissolved in 100ml of water before being made up to 1l with absolute alcohol. This solution was allowed to stand overnight. After standing the solution was titrated against 0.1N oxalic acid using phenolphthalein as an indicator. The colour change was from colourless to pink which allowed a titration value to be determined.

The faecal sample was weighed so that a known weight was used. Distilled water (25ml) was added to the faeces to produce a faecal slurry which was vortexed mixed thoroughly. The slurry was then transferred to a 250 ml conical flask and 95 ml of alcoholic potassium hydroxide was added. Each flask was stoppered with non-absorbent cotton wool and boiled for 20 minutes using a boiling water bath. The flasks were cooled before adding 30 ml 33 % (v/v) hydrochloric acid. Again the flask was cooled and then 50 ml petroleum ether (40°C - 60°C) was added and the mixture was shaken for one minute. The contents of the flasks were allowed to settle and the mixture separated so that the top layer was petroleum ether. Forty mls of this petroleum ether layer was carefully removed by pastette in 10 ml aliquots to small conical flasks. One ml of absolute alcohol containing 0.4% (v/v) amyl alcohol was added and this was titrated against 0.1 N alcoholic sodium hydroxide with thymol blue as indicator.

### 3.10.4 Calculation

In order to determine the faecal fat content of the sample, the following equations were used.

Faecal fat (as fatty acids) grams =

$$\frac{\text{Titration volume} \times \text{Normality NaOH} \times 5 \times \text{Total volume emulsion} \times 284}{25 \times 1000}$$

Faecal fat (as stearic acid) mmol =

$$\frac{\text{Titration volume} \times \text{Normality NaOH} \times 5 \times \text{Total volume emulsion}}{25}$$

### 3.10.5 Validation of method

To determine the accuracy of the method before using faecal samples runs were made using 0.1g stearic acid methyl ester.

#### 3.10.5.1 Standardizing 0.1N alcoholic NaOH

0.1N alcoholic NaOH were titrated against 5ml of oxalic acid

Titration readings of 5.32, 5.49, 5.48 were obtained. This gave an average titration reading of 5.43.

$$5/5.43 \times 0.1 = \text{alcoholic NaOH normality} = 0.092$$

##### 3.10.5.1.1 Determining fat in stearic acid methyl ester sample

Three titration readings of 0.74, 0.72, 0.78 were obtained when titrating 0.1N NaOH against 10ml stearic acid. The average reading was 0.75 ml, this could be used to calculate faecal fat

Faecal fat (as fatty acids) grams =

Titration volume x Normality NaOH x 5 x Total volume emulsion x 284

25 x 1000

⇒ 0.75 x 0.092 x 5 x 284 x 25

25 x 1000

⇒ 0.097g

This was considered an acceptable level to use the method on samples.

### **3.11 DETERMINATION OF RESIDUAL STARCH IN FERMENTATION STUDIES**

Carbohydrate can be classified into monosaccharides, disaccharides and complex carbohydrates. Complex carbohydrates consist of the oligosaccharides (3-10 sugar residues) and polysaccharides (more than 10 sugar residues). Starch is the major polysaccharide of the human diet and is the main storage polysaccharide of dietary staples. Starch consists of two main polysaccharides derived from glucose. Amylose is a linear chain of glucose units with  $\alpha$ 1-4 linkages and amylopectin is a branched chain polymer with 15-30  $\alpha$ 1-4 linked glucose units joined by  $\alpha$ 1-6 linkages.

Starches have been classified on the basis of their digestibility in vitro, giving an indication of the behaviour of starchy foods in the intestinal tract. The amounts of RDS (rapidly digestible starch), SDS (slowly digestible starch) and RS (resistant starch) found in foods varies and depends not only on the source of starch but the type and extent of processing they have undergone. Resistant starch has been defined as 'the sum of starch and products of starch that escape digestion in the small intestine of normal humans (Asp, 1992) and can be divided into types I (physically inaccessible starch), II (resistant starch granules) and III (retrograded amylose). Partly milled grains and seeds are examples of type I resistant starch, raw potato and banana are examples of type II and cooked potato, bread and cornflakes are examples

of type III. The definition is based on physiology of adults and amounts in foods and may therefore differ greatly in infants.

The type of starch available in formulas and infant weaning foods can differ greatly, especially in commercial weaning foods where the processing and sterilising Techniques (often the equivalent of autoclaving) may alter the starch by increasing retrogradation (Björck and Siljeström, 1992). In addition the immaturity of the infant gut may reduce the digestibility of starch that would be readily available in adults. The effect these starches have on the infant *in vivo* is largely unknown but they are likely to reduce the energy value of a food and increase the availability of fermentation substrates in the large intestine.

Resistant starch resists digestion in the small intestine passes into the colon and is available for fermentation by the colonic bacteria. If this escapes fermentation this starch will appear in the faeces. Very little is known about the digestibility of foods in infants. To estimate the amount of starch escaping digestion and fermentation in the infants in the study, the amount of starch appearing in the faeces was determined in a subsample of each group. Again because of the difficulties in collecting 3 or 5 day stool; outputs faecal starch was determined in a single stool output that gave a 'snapshot' of faecal starch excretion.

### **3.11.1 Principle of measurement of resistant starch**

The residual starch in faeces was defined as the sum of the  $\alpha$ -lined glucans which after dispersion can be hydrolysed by  $\alpha$ -amylase and amyloglucosidase.

This method is a modification of the procedure of Englyst, Kingman and Cummings (1992) for the measurement of nutritionally important starch fractions. The faeces were boiled and treated with alkali, the starch is hydrolysed with amyloglucosidase and measured as glucose using a glucose oxidase reagent.

### **3.11.2 Subjects**

A sub-sample of the experimental population was used to investigate faecal starch excretion in infants. Ten exclusively breast-fed infants, 10 exclusively formula-fed infants and 10 mixed fed infants were compared. Faecal samples were collected from these breast-fed and formula-fed infants at pre-weaning (1 - 2 months), early weaning (four weeks after giving solid food), late weaning (9 months - 1 year) and very late weaning (18 months - 2 years). Faecal samples from mixed fed infants were collected at early, late and very late weaning. Samples were collected within one hour of defaecation and scraped from the nappy into a universal container. This was then stored at -20°C for later analysis of faecal starch.

### **3.11.3 Faecal starch analysis**

A single aliquot of faeces of 1.6g was collected from these infants at each development stage. Faecal starch analysis was carried out in batches so the samples were stored at -20°C until required for the assay.

Frozen faeces previously stored at -20°C was allowed to thaw at room temperature. A 1.6% (1.6g/l) faecal slurry was made in 10 mls buffer pH 7.0. A blank tube containing just buffer solution and tubes containing a known quantity of a reference starch were treated the same as experimental tubes. All tubes were placed into a rapidly boiling water bath for 30 minutes. The tubes were then cooled by standing them in a bath of ice water for at least 30 minutes. An equal quantity (10ml) of 4M potassium hydroxide was added to the tubes and they were vortexed mixed. Tubes were kept chilled for 15 minutes regularly vortex mixing. A second set of tubes corresponding to the test samples, blanks and standards was prepared containing 10 ml of 0.5 M acetic acid. The first set of tubes were removed from the ice water and 1 ml of the contents was added to the corresponding tube and were mixed well. Amyloglucosidase (0.2 ml) was added before mixing and placing in a water bath at

70°C for 30 minutes. Immediately the tubes were placed in a boiling water bath for 10 minutes. The tubes were then cooled to room temperature and the pH was adjusted by adding 0.6 ml of 5 M potassium hydroxide. The tubes were mixed and centrifuged to obtain a clear supernatant.

#### **3.11.4 Determination of residual starch**

A GOD-PEROD kit (Boehringer Mannheim) was used to determine glucose. 200 µl of blanks, standards and samples were placed into labelled tubes and 5 ml of GOD-PEROD reagent was added to these. The tubes were left for 10 minutes at room temperature before absorbance was measured by spectrophotometer at 610 nm. The tubes were measured against the blank.

#### **3.11.5 Calculation**

Residual starch in g/100g of faeces is given by:

$$\frac{A_t \times V_t \times C \times D \times 100 \times 0.9}{A_s \times W_t}$$

As x Wt

where At = absorbance of test solution

Vt = volume of test solution (here 10)

C = concentration (in mg/ml of the standard used (here 2.5)

As = absorbance of standard

Wt = weight in mg of substrate used

D is a dilution factor (here 1)

0.9 is a factor to convert monosaccharides to polysaccharides

#### **3.11.6 Validation of method**

The method was first validated using reference samples of wheat flour and raw potato starch from a testing kit (product no. 61-000, Englyst Carbohydrate Services Limited,

UK). This was carried out to gain familiarity with the experimental procedure and to resolve any problems that arose prior to analysing infant samples. As the reference starches had known values these were measured and conditions changed until accurate results were routinely obtained.

### **3.12 STATISTICAL ANALYSES**

SCFA data was not normally distributed so non-parametric tests were used. In the case of the characteristics of the sample where data was normally distributed two-sample t-tests were used or in the case of proportions chi-square test. All statistical analyses were carried out using Minitab and advice was sought from a statistician.

#### **3.12.1 One-sample Wilcoxon test**

For longitudinal analysis where data was not normally distributed one-sample Wilcoxon test.

#### **3.12.2 Kruskal-wallis and Mann-Whitney t-test**

For comparisons between the three feeding groups where data was not normally distributed a Kruskal-Wallis test was used. If the p-value given was significant, Mann-Whitney non-parametric test was then used.

#### **3.12.3 Two-sample t-test**

A two sample t-test was carried out where data was normally distributed.

#### **3.12.4 Chi-square test**

Where proportions in samples were to be compared a Chi-square test was carried out.



## **Chapter 4**

### **Characteristics of Sample in Longitudinal Study**

This chapter will describe the characteristics of the 3 infant feeding groups, breast-fed, formula-fed and mixed fed, used in the longitudinal study.

#### **4.1 THE SAMPLE SIZE**

A power equation (Altman, 1980) utilising data obtained in a pilot study (described in Chapter 2) was used to determine the sample size needed to investigate the difference in concentration of SCFA produced by in vitro fermentation between three groups of infants. In the pilot study the standard deviation for total SCFA was 10  $\mu\text{mol}/\text{ml}$ . To detect a difference of 15  $\mu\text{mol}/\text{ml}$ , the power equation concluded that 25 infants in each group were needed. It was decided to recruit 50 infants to allow for 50% dropout, which was the dropout rate seen in previous studies.

As this was a longitudinal study, it was necessary to recruit infants within a limited timeframe to allow samples to be obtained at the time-points previously chosen. This meant that the 150 infants required would have to be recruited over two years. As the collection of samples from each subject was quite intensive in the first few months, recruitment had to be carried out at intervals during this two-year period. An initial batch of infants were recruited and samples were collected from them at one month, two months and during weaning (4 weeks after beginning solids). Another batch of infants were then recruited and the first set of samples were collected from them before a further set of subjects were recruited and so on. In this way the intensive period of collection of samples was completed before more subjects were recruited, which occurred roughly every 4 months.

#### **4.2 SAMPLING FRAME**

As samples had to be collected from subjects within one hour of defaecation, the geographical area that mothers could be recruited from was restricted. Mothers were recruited from North and West Glasgow so that travelling to obtain the

sample and return to the laboratory was kept to a maximum of 30-45 minutes. The area “North and West Glasgow”, already used in a previous study in the department, had been shown to be representative of Glasgow as a whole (Savage, 1998).

Postal codes, which were used to recruit the sample, were as follows:

**G3, G11, G12, G13, G14, G15, G20, G61, G62 and G81**

**Table 4.1 Postcodes from the sample and associated neighbourhood type**

Neighbourhood Type <sup>*</sup>	Postal code
1	G61.1, G61.2, G61.3, G61.4, G62.6, G62.7, G62.8
2	G81.6
3	G11.5, G11.7, G12.0, G13.1
4	G13.2, G13.3, G13.4, G14.9, G15.6, G20.0, G81.3, G81.4
5	G81.1, G81.2, G81.5
6	G3.6, G3.7, G12.8, G12.9, G20.6
7	G15.7, G15.8
8	G3.8, G11.6, G14.0, G20.7, G20.8, G20.9

\* see page 129, section 4.4.1

Recruitment was therefore restricted to a single maternity hospital, The Queen Mother’s Hospital, which served these postal code areas. Ethical approval for the study was obtained from Yorkhill NHS Trust. Suitable mother and infant pairs were selected from maternity wing records and invited to participate in the study. Only wards dealing with normal deliveries were used. Mothers who agreed to participate gave written, informed consent.

A total of 299 mothers were approached to participate in the study. This involved speaking to mothers shortly after they had given birth. The study was described in detail and mothers were left with a leaflet explaining all aspects of the study. Although the leaflet had a telephone number to contact if mothers wished to take part, it was found that this was not used. The only practical way of recruiting, therefore, was to revisit the mother before she left the maternity ward to ask if she wanted to take part in the study.

Of the 291 mothers who were approached regarding the study 118 left hospital before a second visit could be made. Of the remainder, 46 mothers opted not to take part in the study and 127 mothers agreed to take part. Within the group of mothers that agreed to take part, there were three subgroups; those who participated fully throughout the study (63), those who gave some samples but not enough to be followed longitudinally (23) and those who agreed to take part but never gave samples (41).

#### **4.2.1 Exclusion criteria**

Infants had to be healthy and full-term. No infants under birthweight 2250g were included in the study. If the infant had been ill at birth, or was shown to have any problems, mothers were not approached. Mothers who did not have access to a telephone at home could not take part as it was an integral part of the study to call the department as soon as a dirty nappy was obtained so that it could be collected quickly. Finally because of the need to collect samples quickly mothers were excluded if they were outwith the recruitment area (within 60 minutes round trip).

#### **4.2.2 Problems with recruitment and collection of samples**

At the time of this study, the maternity hospital was taking part in a breast-feeding initiative to encourage mothers to breast-feed. Because of this it was difficult to recruit mothers who were exclusively formula feeding and consequently the number in this group are lower than we had hoped for.

Another problem with recruitment was that some mothers thought the collection of dirty nappies a strange request and did not wish to participate. There was no benefit to the mothers in agreeing to take part in this study and so many mothers refused. In addition, in recent years the length of stay in the maternity hospital after birth has been dramatically reduced. Some mothers stay for one or two days, others leave a few hours after giving birth. This makes it very difficult to visit mothers twice during their stay, once to explain the study and on a second

occasion to secure their recruitment. It became clear early in the project that if the mothers were not visited a second time they were lost to the study.

Once recruitment was underway, it was difficult to collect samples and recruit at the same time. The routine of the maternity wards meant that mothers could be visited only at certain times (to avoid ward round or relative's visits) and hence if a sample needed to be collected at this time, it was necessary to forgo the ward visit. As far as possible the same researcher saw mothers in the hospital and collected samples to encourage continued participation.

Breast-fed infants prior to weaning produce very small faecal samples. This made it very difficult to get enough samples from all infants to carry out the assays. Although up to three nappies were requested to cover each development stage, there was often still not enough sample but asking for more nappies than this was found to be intrusive and off-putting for the mothers. There were other problems with collecting samples at individual development stages. Although some leeway was given around the date of sample collection, there were missing points because the infant did not defaecate at a convenient time. Many infants produced samples in the evening and at night when collection was not possible. The sample was required to be as fresh as possible so it was stipulated that the first morning nappy was unsuitable as mothers could not be sure when defaecation took place.

We tried to overcome the problem of mothers returning to work by collecting from childminders and nurseries. This was not always possible if they were outwith the geographical area where collection was feasible.

#### **4.3 DESCRIPTION OF THE SAMPLE**

The mothers who were approached to take part in the study were classified into 4 groups as follows:

Mothers who participated throughout study	Participants 1
Mothers who did not give enough samples to study longitudinally	Participants 2
Mothers who said yes but did not give samples	Non-participants 1
Mothers who said no to taking part	Non-participants 2

#### **4.3.1. Comparison of participants and non-participants**

These four groups were investigated to see if there was a difference between those who participated in the study and those who did not. The data of each group were compared for feeding practice, sex of infant, birthweight of infant, mother's previous live births, mother's previous non-live births, age of mother and postcode (for deprivation score).

#### **4.3.2 Feeding practice**

Feeding practice was divided into three groups, exclusively breast-feeding, exclusively formula-feeding and mixed feeding. Exclusively breast-feeding was defined as giving no other solid or liquid (with the exception of water) other than breast-milk prior to the beginning of weaning. Exclusively formula-feeding was defined as giving only formula-milk prior to weaning. The formula-milk given was chosen by the mothers without intervention or influence and mothers could freely swap between formula-milks.

Mixed feeding was a mixture of breast and formula-milk in various quantities encompassing those who had begun breast-feeding and then changed to formula feeding through to those who were breast-fed but had had a single bottle of formula-milk. (Table 4.2).

Only infants who gave samples could be assigned to one of three feeding groups. The non-participants 1 and 2 could be assigned only as breast or formula-fed as information on this group was obtained shortly after birth. All infants were assigned a 1 or a 2 for breast-fed and formula-fed respectively to compare between participants and non-participants by chi-squared test. All mixed-fed

infants were assigned to the breast-feeding group as they would have started as breast feeders.

**Table 4.2 Description of feeding characteristics of mixed fed infants**

No	Description of feeding
1	Breast-fed exclusively except a single bottle at two months.
2	Breast-fed for 10- 11 days then exclusively formula-fed.
4	Breast-fed for 2-2.5 weeks then exclusively formula-fed.
1	Breast-fed for 5 weeks then exclusively formula-fed.
2	Mixture of breast and formula-milk from birth.
1	Breast-fed exclusively for two months then additional occasional bottle (max 1/wk).
1	Exclusively breast-fed for one month then breast-fed morning and evening feeds with formula milk in between.
1	Exclusively breast-fed for two months then 4 breast-feeds / day plus 12-15 fl oz formula milk

#### 4.3.3 Sex of infant

Infants were classified 1 for males and 2 for females for comparison by chi-squared test.

#### 4.3.4 Deprivation score

A Carstairs Deprivation Category (DepCat) was assigned using postal codes including the first digit from the second part (eg G3 5 or G81 7) (Carstairs and Morris, 1990). The Carstairs deprivation categories are a way of characterising the degree of deprivation in each area. To calculate the deprivation score a ranking technique is used, based upon the values of an unweighted combination of four standardised census variables. These variables are: male unemployment, no

car, overcrowded housing, low social class. These variables are thought to be representative of material disadvantage. Demographic variables are omitted from this analysis as they are considered not to indicate deprivation.

The resultant values give categories 1-7 where 1 is the most affluent and 7 is the most deprived areas. A chi-squared test was used where a category of 1 was given to DepCat scores 1-3 and a 2 to DepCat 4-7.

#### **4.3.5 Previous live and non-live births.**

This information was obtained from the hospital records of each mother. A code of 1 was assigned to mothers who had had any live births and 2 to those who had no previous live births. This categorisation was also used for non-live births allowing both variables to be tested by chi-squared test.

#### **4.3.6 Age of mother**

The age of mother was divided into two groups; less than 30 years of age (1) and more than 30 years of age (2) and then compared by chi-squared test.

Chi-square tests for all the factors discussed above are shown in Table 4.3 and p-values are given. The number of subjects that fall into category 1 (for each factor) are shown as a proportion of total number of subjects in each group. Category 1 is breast-feeding, male, DepCat score 1-3, any previous live births, any previous non-live births, mother's age less than 30.

There were significantly more affluent mothers who participated (Participants 1 and 2) than among the non-participants (1 and 2). There was a significantly higher proportion of mothers breast-feeding in the two participant groups than in the non-participant groups and significantly more mothers were under 30 years of age in the non-participant groups.



**Table 4.3 Comparison of characteristics of participants and non-participants.**

	Number in category 1/total number				p-value
	Participants	Participants	Non-	Non-	
	1	2	participants 1	participants 2	
	n = 63	n = 23	n = 41	n = 46	
Feeding	47/63	17/23	21/41*	22/45**†	0.012
Sex of infant	21/63	13/23	18/41	19/42	0.243
DepCat score	31/63	9/23	8/41**	5/43****†	0.000
Previous live births	20/43	6/15	15/29	6/24	0.227
Previous non-live births	7/43	4/15	8/29	5/24	0.667
Age of mother	14/41	9/15	20/29**	15/24*	0.019

\* p<0.05, \*\* p<0.01, \*\*\* p<0.01 when compared with participants 1

† p<0.05, †† p<0.01 when compared with participants 2

For the factors where a significant difference had been seen, the total participants (Participants 1 and 2) were compared with total non-participants (non-participants 1 and 2). The ratios are breast-feeding, mother's age less than 30 and DepCat score 1-3 as a proportion of total subjects in each category. Table 4.4 shows that all the significant differences previously seen are still present when comparing participants with non-participants.

**Table 4.4 Comparison of characteristics for all participants and all non-participants.**

	Number in category 1/total number		p-value
	Participants	Non-participants	
	n = 86	n = 87	
Feeding of infant	64/86	43/86	0.001
Age of mother	23/56	35/53	0.009
DepCat score	40/86	13/84	0.000

#### 4.3.7 Birthweight of infant, age of mother

Subjects were combined to give 2 groups; participants and non-participants and compared by two-sample t-test for the above variables that were normally

distributed. There was no significant difference in the birthweight of infants between participants and non-participants. Mothers who participated were significantly older than those who refused (Table 4.5).

**Table 4.5 Comparison of birthweight of infant and age of mother for participants and non-participants.**

	Number in category 1/total number				p-value
	Participants		Non-Participants		
	n =86		n = 87		
	Mean	SD	Mean	SD	
Birthweight	3.41	0.50	3.35	0.48	0.44
Age of mother	30.7	4.75	27.1	6.1	0.001

#### 4.4 COMPARISONS OF BREAST-FED, FORMULA-FED AND MIXED FED INFANTS

##### 4.4.1 Neighbourhood Type

As well as comparison by Depcat score, infants were compared by neighbourhood type (Table 4.6). This is a categorisation using postcodes (in the same way as with DepCat scores) to classify different types of neighbourhood by deprivation. The neighbourhood type score is a similar characterisation as DepCat. Similar areas are grouped together in 'neighbourhood types'. In contrast to DepCat, demographic variables are taken into account and, as opposed to being based on four census variables, thirty socio-demographic factors were analysed to divide the Greater Glasgow Health Board region into 8 categories which describe these areas in terms of mix of housing and socio-economic factors. The most affluent area is categorised 1 and the least affluent is 8. For the purpose of the chi-squared test, comparisons were made between those in categories 1-3 against those in 4-8.

Neighbourhood type also allowed the sample to be compared with the Greater Glasgow Health Board area. The subjects in this study did not completely match the population of Glasgow (Table 4.6). This is a problem of conducting studies, such as this, where there is reliance on subjects to take part and co-operate. This

immediately leads to those who are more motivated taking part and they tend to be of higher social class. If the neighbourhood type categories are paired (e.g. 1 and 2, 3 and 4) it can be seen that the categories 3-6 contain approximately the same percentages as the population of the Greater Glasgow Health Board. The highest social classes (1 and 2) are better represented than in the general population and the lower social classes (7 and 8) are less well represented than in the general population.

**Table 4.6 Comparison of neighbourhood type between the subjects in this study and the population of the Greater Glasgow Health Board (GGHB) area.**

Neighbourhood Type	No.	%	%GGHB
1	21	33	13
2	1	2	9
3	11	17	10
4	11	17	17
5	3	5	20
6	11	17	5
7	0	0	10
8	5	8	17

#### 4.4.2 Age at weaning

There was no difference in age of the start of weaning between the three infant feeding groups (Table 4.7, Table 4.8). For the chi-squared test, the age of weaning was split into less than (category 1) and more than 16 weeks (category 2). Other categories analysed were as described earlier in this Chapter.

Figure 2.1 shows the weaning age for all infants and also for infants in each feeding group. The weaning age of infants in the Glasgow Infant Growth Study are shown for comparison.

No differences were seen between the feeding groups for any of the variables when compared by chi-square test.

**Table 4.7 Comparison of characteristics of breast-fed, formula-fed and mixed fed infants.**

	Number in category 1/ total number		
	Breast-fed	Formula-fed	Mixed Fed
	n =28	n = 16	n = 19
Sex of infant	8/28	4/16	9/19
Age at weaning	10/28	9/16	9/18*
Neighbourhood type	16/27*	5/16	11/19
DepCat score	17/28	5/16	10/19
Previous live births	11/20*	4/8*	5/15*
Previous non-live births	4/20*	1/8*	2/15*
Age of mother	4/18*	3/8*	7/15*

\* Some data was not available

#### 4.4.3 Birthweight and age of mother

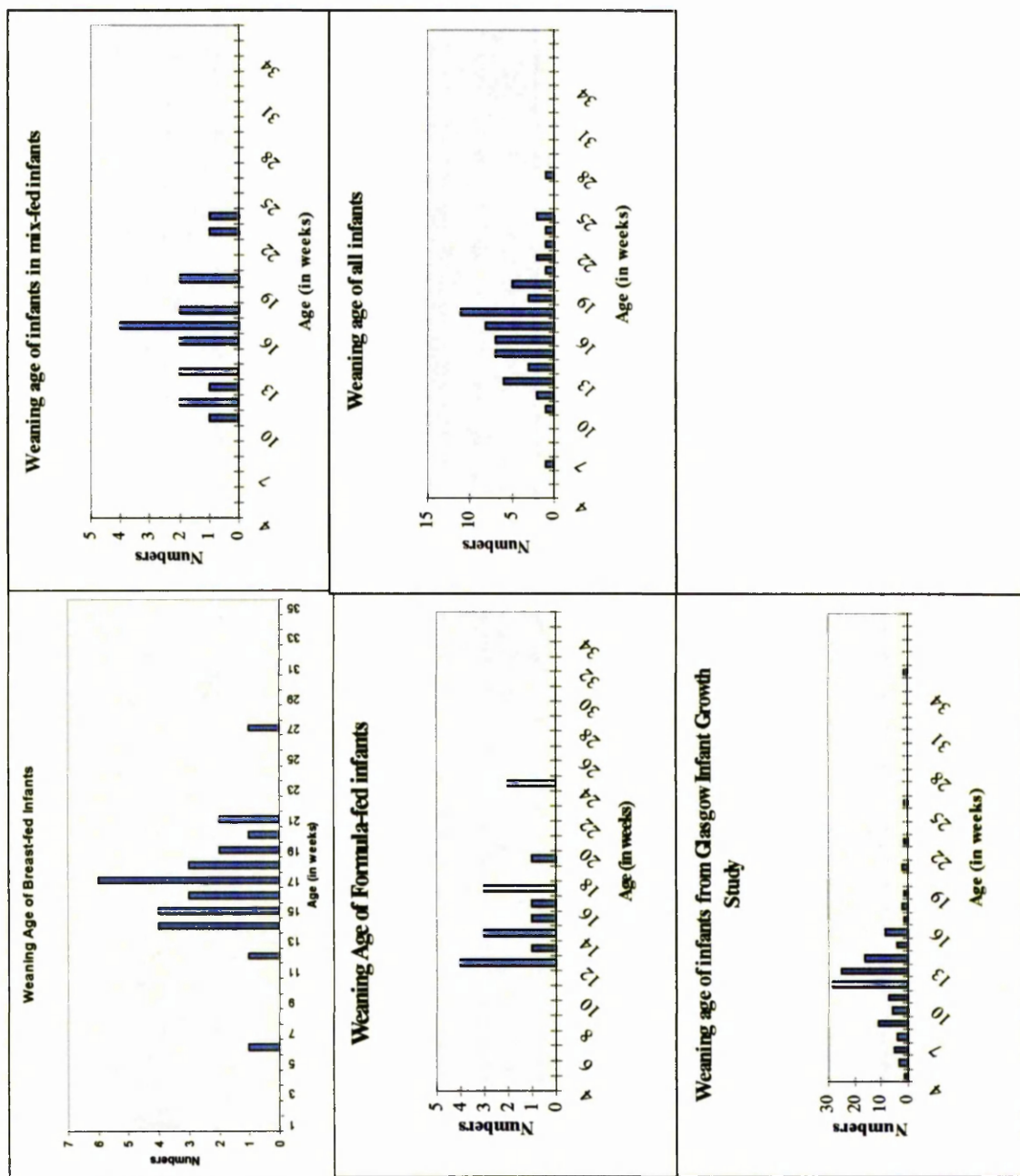
The birthweight of the infant, age of mother and age at weaning were also compared (Table 4.8) by two-sample t-test. Breast-fed infants were significantly lighter at birth than the mixed fed infants but there was no difference compared with the formula-fed infants.

**Table 4.8 Comparisons of birthweight of the infant, age of mother and age at weaning between infant feeding groups.**

	Breast-fed		Formula-fed		Mixed Fed	
	n = 28		n =16		n =19	
	Mean	SD	Mean	SD	Mean	SD
Birthweight of infant	3.31*	0.52	3.65	0.55	3.63	0.40
Age of mother	32.2	3.88	30.4	2.50	31.2	4.84
Age at weaning	16.6	3.50	15.7	3.86	18.5	12.9

\*p < 0.05 compared with mixed fed infants.

Figure 4.1 Age of start of weaning for infant groups



## 4.5 DIETARY DATA

Information on the first weaning foods given to each infant was gathered. However, as the numbers of infants were small the data can be compared only qualitatively (Table 4.9 and 4.10). Breast fed and mixed fed infants were more likely to be given baby rice than formula infants who, as a group, were fed a greater variety of foods at the start of weaning. Figure 2.2 shows the first weaning food given for all infants and infants in each feeding group. First weaning foods for the Glasgow Infant Growth Study (Savage, 1998) is also shown.

**Table 4.9 Comparisons of first weaning food given between infant feeding groups.**

		Number of Infants			
		Breast-fed	Formula-fed	Mixed fed	All Infants
		n = 28	n = 16	n = 19	n = 63
1	Vegetables	2	4	2	8
2	Fruit	4	0	0	4
3	Baby cereal	1	1	1	3
4	Baby rice	16	3	8	27
5	Rusk	0	1	0	1
6	Commercial baby food	3	4	4	11
	No data	2	3	4	9

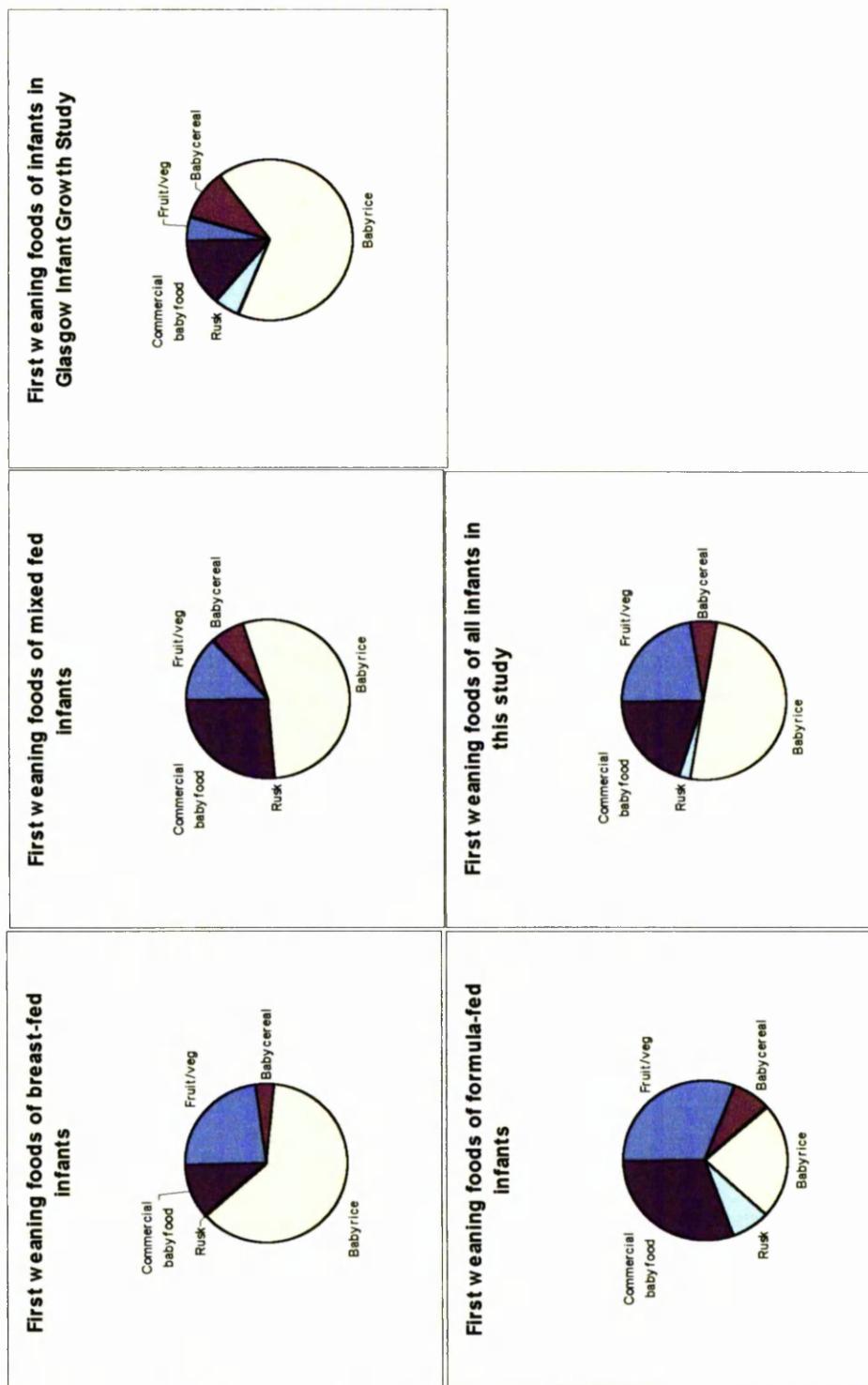


Figure 4.2 First weaning foods given in infants

**Table 4.10 Comparisons of first weaning food given between infant feeding groups when fruit and vegetables are grouped together.**

		Number of Infants			
		Breast-fed	Formula-fed	Mixed fed	All Infants
		n = 28	n = 16	n = 19	n = 63
1	Fruit & Vegetables	6	4	2	12
2	Baby cereal	1	1	1	3
3	Baby rice	16	3	8	27
4	Rusk	0	1	0	1
5	Commercial baby food	3	4	4	11
6	No data	2	3	4	9

#### 4.6 DIET DURING WEANING

As the faecal microflora is highly likely to be influenced by the weaning diet, information was collected of the foods that each infant had eaten on the three days prior to giving each sample. In an ideal study, detailed records of dietary intake would have been collected. However this was not feasible given only one researcher. A detailed questionnaire was impractical because of the time limitations when the sample was collected. Therefore, mothers were asked to recall a three-day dietary intake for each infant prior to giving a sample. The information obtained ranged from very brief to very detailed dietary histories, and consequently was of little real value. In future studies funding should include costs of detailed dietary analysis.

Table 4.11 shows the range of milks given by mothers in the study. There are some composition differences between milks but mothers were not restricted in which formula they chose to use.



**Table 4.11 Formula milks used by mothers throughout the study**

<b>1 month</b>	<b>2 months</b>	<b>Early Weaning</b>	<b>7 months</b>	<b>9 months</b>	<b>1 year</b>
Aptamil	Aptamil		Aptamil		Aptamil
Milumil	Milumil	Milumil	Milumil	Milumil	Milumil
Cow & Gate premium	Cow & Gate premium	Cow & Gate premium	Cow & Gate premium	Cow & Gate premium	
	Cow & Gate plus	Cow & Gate plus			
SMA Gold	SMA Gold	SMA Gold	SMA Gold		
SMA White	SMA White	SMA White	SMA White	SMA White	SMA White
			SMA Progress	SMA Progress	SMA Progress
Farleys First	Farleys First	Farleys First			
Farleys	Farleys	Farleys	Farleys	Farleys Second	Farleys
Second	Second	Second	Second		Second
			Farleys Follow-on	Farleys Follow-on	Farleys Follow-on
				Boots Follow-on (vanilla or strawberry)	
				Cows milk	Cows milk

Table 4.12 indicates the food given by each mother at each development stage. A typical day's menu is given for each infant if the information was given. The information given does not allow further analysis. Food given at late weaning was generally described as normal family diet and therefore is not included in the tables. One of the problems of asking mothers to recall diet history without asking specific questions is that they may leave out foods that they consider to be not healthy.

The information in Table 4.12 is the food generally given at early weaning and therefore is not necessarily the same as the first weaning food given. In the breast-fed group infants generally received baby rice, cereal or fruit and vegetables. Only a few mothers gave commercial foods as opposed to homemade. In contrast in both the formula-fed and mixed-fed group, half of the mothers gave commercial products.

Table 4.13 and 4.14 give an indication of starch and fibre content of foods given by mothers. The amount of starchy or high fibre foods given, especially, in early weaning may have an effect on the bacterial colonisation of the gut and the profile of faecal SCFA produced. The fibre intake in all groups of infants increased at

late weaning with cereal foods such as weetabix, readybrek and muesli being given more often.

Foods such as banana, cornflakes or mashed potato that have been left to cool may contain significant quantities of resistant starch. Resistant starch may be higher in some commercial foods because of the methods used during processing eg., autoclaving. Complex carbohydrates may be difficult for the young infant to ferment and this could lead to an increase in stool output and possible faecal energy losses.

**Table 4.12 Foods consumed at each development stage**

Subject	PW	Early weaning	Late weaning
<b>Breast-Fed</b>			
1	BF	Pureed potato and carrot Mashed banana	Milupa Sunshine Orange Pureed potato and carrot, apple Weetabix
2	BF	Milupa sunshine orange Spaghetti bolognese (H/M), stewed fruit	Mince and potatoes (H/M) Cauliflower cheese (H/M), banana Weetabix
3	BF	Baby rice, stewed fruit, fromage frais	Pasta and vegetables, yoghurt Chicken, potatoes, vegetables, custard, fruit juice
4	BF	Baby rice, Organix cereal Pureed vegetables	Milupa Sunshine orange Mashed fishfingers and peas Pureed pork and vegetables
5	BF	Baby rice, pureed vegetables	1 x Heinz tin
6	BF	Baby rice, pureed vegetables or fruit	Toast, fromage frais, fruit Weetabix
7	BF	2 teaspoons of Heinz jar twice/ day	Cauliflower cheese (H/M), fruit
8	BF	Baby rice, Milupa sunshine orange	Meat and vegetables (H/M), fromage frais
9	BF	Baby muesli Pureed fruit, pureed vegetables, low sugar rusk	Normal family diet No Data
10	BF	Baby rice and grated apple 1 jar Organix summer vegetables	Milupa sunshine orange Mashed savoury dish and sweet dish (H/M) twice/ day, grated apple
11	BF	Pureed fruit or vegetables twice/ day	Cornflakes with cows milk Macaroni cheese (H/M), mashed fruit Greek yoghurt and tinned mandarins, unsweetened juice Readybrek
12	BF	Baby rice and ½ mashed banana	Macaroni cheese Pasta and vegetables, fruit No Data

13	BF	Pureed vegetables, mashed banana Pureed vegetables, Heinz yoghurt Farley's creamy oat porridge Pureed vegetables, St. Ivel apple puree, low sugar rusk Pureed vegetables, ½ mashed banana No Data 3 x pureed fruit/ day, baby juice	Hot oat cereal, fruit, yoghurt Lamb stew and couscous (H/M), bread, fruit Baby yoghurt, mashed banana No Data Cereal, juice Lentil soup Cheese, beans and potatoes, fruit Beef casserole, dairy tea cheese, rich tea biscuit Stewed fruit, semolina 2 tins vegetable puree 1 x Farley's teatime treat/ day, fruit Weetabix Cheese sandwich, yoghurt, banana Fishfingers, cheese, potatoes and beans, yoghurt No Data Cereal Pureed meat and vegetables, pureed fruit or yoghurt twice/ day No Data Readybrek, banana Cauliflower cheese, mixed fruit Turkey, potatoes and vegetables Pureed fruit, pureed vegetables, fromage fraise Heinz chocolate pudding 1 x Heinz jar/ day, bread, juice Weetabix Soup, toast, fromage fraise Meat, potatoes, vegetables (H/M) No Data
14	BF		
15	BF		
16	BF	Stewed fruit	
17	BF	2 x baby rice and pureed fruit/ day	
18	BF	Fruit and vegetable purees (H/M), fruit juice	
19	BF	Baby cereal, pureed vegetables and fruit	
20	BF	Pureed vegetables or fruit with baby rice once or twice/day, rusk	
21	BF	No Data	
22	BF	Baby cereal Pureed fruit, pureed vegetables twice/ day	
23	BF	Baby rice, pureed fruit	
24	BF	1 x Heinz jar/ day	
25	BF	Heinz/ Cow & Gate jars	
26	BF	Baby rice Sweet potato and carrot mashed Farley's banana and custard	
27	BF	Baby rice and pureed fruit or vegetables twice/ day	Cereal Pureed meat and vegetables 3 x / day, fruit

28	BF	Baby porridge 1 x jar stewed fruit	Boot's muesli Savoury dish and sweet dish (commercial) Cow & Gate hotpot, fromage fraise, banana
<b>Formula-Fed</b>			
1	FF	Baby porridge mashed potato & carrot (H/M)	Readybrek with cow's milk Baked beans on toast, fromage fraise, apple
2	FF	Boots Spring vegetable Readybrek Heinz tin ( variety) for lunch and tea	Chicken pie, potato, carrot, yoghurt Weetabix ¾ Heinz tin, toast and butter ¾ Heinz tin, yoghurt, fruit
3	FF	Baby rice and pureed fruit	Banana, cereal, toast Soup and bread Normal family diet (chopped), orange juice
4	FF	Baby rice, pureed fruit Rusk, yoghurt	Banana , toast Brown bread, cheese, milky bar Chicken and pasta casserole (commercial), custard Orange juice
5	FF	Boot's muesli, stewed apple	Boot's muesli Pureed meat, potato and vegetables Stewed fruit, Apple juice
6	FF	Baby rice, Milupa cereal Pureed vegetables, fromage fraise	Cereal Heinz jar, fruit Meat and vegetables (H/M), yoghurt No Data
7	FF	Oats and apple muesli Spaghetti bolognese (commercial, dried)	
8	FF	3 x solid meal/day (commercial)	
9	FF	Milupa sunshine orange 1 x Heinz jar (sweet)	Weetabix, plain biscuit, Cow & Gate juice Milupa cheese, tomato and pasta, yoghurt, fruit Milupa packet baby food, toast, yoghurt Baby porridge Heinz jar, mashed banana Heinz jar, fruit, yoghurt Baby cereal Bread, butter, cheese, yoghurt
10	FF	1 x meal/ day pureed fruit or vegetables (H/M)	



11	MF	Baby rice and fruit Pureed vegetables, yoghurt Mashed vegetables, mashed fruit 2 x Farley's packets Milupa sunshine orange 2 x commercial jars 3 x Milupa packets	No Data
12	MF		2 x commercial jars
13	MF		Baby cereal Spaghetti bolognese (H/M), yoghurt Fisherman's pie, banana, orange Weetabix and toast
14	MF		Macaroni cheese (commercial but not baby variety) Spaghetti and sausage, yoghurt, crisps, ½ tea biscuit, fruit, bread and butter
15	MF	½ Heinz jar, few spoonfuls of cereal	Weetabix, ½ banana, fromage frais Normal family diet at tea time
16	MF	Pureed vegetables	3 x meals/ day
17	MF	Cow & Gate baby rice, baby porridge Cow & Gate raspberry and peach Milupa rice and orange, Cow & Gate chicken and vegetables Baby rice, pureed fruit, rusks, custard	Rusk, cereal Lentil soup and bread Spaghetti bolognese, vegetables, juice Grilled sausage, scrambled egg, toast Mashed potato and boiled egg, yoghurt Pasta with tomato, Heinz chocolate pudding 3 x meals/ day, family food, well mashed
18	MF		
19	MF	3 x pureed fruit or vegetables/ day	
H/M Homemade			

**Table 4.13 Starch and fibre values (g/ 100g wet wt) of commercial foods consumed by infants**

Product	Starch	Fibre
<b>HEINZ based on foods available 1998/1999</b>		
Creamed porridge	5.2	0.4
Apple and banana	7.0	0.9
Mixed fruit muesli	6.7	0.5
Porridge oats with prunes	5.2	0.7
Spaghetti bolognese	8.0	0.5
Apricot custard	4.7	0.3
Banana delight	4.9	0.2
Chocolate pudding	4.4	0.3
Crème caramel	4.7	0.0
<b>FARLEY based on foods available 1998/1999</b>		
<b>First Timers</b>		
Pure baby rice*	85.6	2.0
<b>Breakfasts</b>		
Fruit and yoghurt	20.5	1.1
Muesli	21.9	2.1
<b>Tea Timers</b>		
Cheese and vegetable bake	40.2	1.8
Cauliflower and broccoli cheese	40.0	1.6
Country vegetables and turkey	43.3	2.1
Farmhouse vegetables and chicken	43.5	2.3
Lancashire hotpot	45.6	2.0
Leek and potato pie	33.9	1.6
<b>Farley's junior choice</b>		
Fruity muesli	25.7	3.1
Pear and apple cereal	26.7	3.6
Cheesy vegetable pasta	43.8	2.2
Shepherds pie with lamb	43.0	2.6
Chicken and mushroom supreme	39.0	1.9
<b>COW &amp; GATE based on foods available 2000</b>		
Banana rice pudding	6.0	Trace
Chicken and vegetables	9.0	1.3
<b>MILUPA based on foods available 2000</b>		
Sunshine orange	36.4(per 100g powder)	0.6

Fibre measured by Englyst method.

\*g/100g dry weight



**Table 4.14 Starch and fibre values (g/ 100g wet wt) of foods consumed by infants**

Product	Starch	Fibre (by Southgate)	Fibre (by Englyst)
Spaghetti (white, boiled)	21.7	1.8	1.2
Macaroni (white, boiled)	18.2	1.5	0.9
Lentils(green, brown & whole, boiled in unsalted water	16.2	3.3	1.9
Weetabix	70.5	11.6	9.7
Cornflakes	77.7	3.4	0.9
Rice Krispies	79.1	1.1	0.7
Muesli, Swiss style	46.0	8.1	6.4
With no added sugar	51.4	10.5	7.6
Ready Brek	66.9	N	7.2
Brown bread average	41.3	5.9	3.5
toasted	52.1	7.1	4.5
White bread average	46.7	3.8	1.5
toasted	53.4	4.5	1.8
Banana	1.5	2.0	0.7
New potato, average, boiled for 20 min in unsalted water	14.8	1.3	1.0
Old potato, average (boiled as above)	16.3	1.4 *	1.2
Potato mashed with butter/margarine	14.5	1.3	1.1
Peas, boiled in unsalted water	7.6	4.7	4.5
Canned, re-heated, drained	6.3	5.7	5.1
Broccoli, boiled in unsalted water	Trace	N	2.3
Carrots, old, boiled in unsalted water for 12.5 min	0.2	2.8	2.5
Carrots young, boiled as above	0.2	2.7	2.3
Tomato, raw	Trace	1.3	1.0
Turnip, boiled in unsalted water	0.1	2.0	1.9
Sweetcorn, baby, canned, drained	0.6	N	1.5
Corn-on-the-cob, boiled in unsalted water	10.0	2.5	1.3
Baked beans, canned in tomato sauce, re-heated	9.4	6.9	3.7
Cheese sauce (whole/ semi-skimmed milk)**	4.6	0.2	0.2

Taken from McCance and Widdowson, 1992 (\*\* from recipe).

N Nutrient is available in significant quantities but there is no reliable info on the amount

\* Analysis showed resistant starch present at 0.7 g per 100g in boiled potatoes kept at room temperature for 2 hours

## 4.7 CONCLUSIONS

From the basic data collected from mothers and infants we were able to compare the participants with non-participants and also differences between the three feeding groups.

The only difference in infants seen between the feeding groups was birth weight. The birth-weights of breast-fed infants were slightly lower than that of both formula and mixed fed infants but this reached significance only between breast and mixed fed infants. There were no differences between social class based on Neighbourhood type or DepCat scores in the three groups. As the characteristics of the feeding groups were similar, comparison of faecal SCFA and fermentation capacities can be made on the basis of feeding practice alone.

There were differences between maternal characteristics of participants and non-participants. Participants were of higher social class, mothers were older and infants were more likely to be breast-fed than non-participants. Participants, when compared with Glasgow as a whole for neighbourhood type, were not representative of the area of Glasgow. These differences were unavoidable due to the nature of the study. Although the mothers and infants taking part may not be representative of Glasgow these differences will not interfere with the aims of the study which were to compare the effects of infant feeding on the development of the intestinal microflora.

## **Chapter 5**

### **Changes in Faecal Short Chain Fatty Acids of Breast-Fed, Formula-Fed and Mixed Fed Infants in the First 18 Months of Life**

## 5.1 INTRODUCTION

This chapter describes the faecal SCFA profile of the three infant feeding groups, breast-fed, formula-fed and mixed fed, from birth to eighteen months.

It is well established that the faeces of breast-fed and formula-fed infants differ in many aspects. These include colour, consistency, odour (Weaver *et al.*, 1988b) and the composition of the faecal flora (Edwards *et al.*, 1994). Breast-fed infants tend to have bifidobacteria and bacteroides as predominant organisms with lower amounts of *Enterobacteriaceae* and bacteroides species. In contrast, formula-fed infants have a faecal flora that resembles the adult more closely, tending to have more *Enterobacteriaceae*, bacteroides and streptococci species. The differences in flora determine the pattern of carbohydrate fermentation which is reflected in the faecal short chain fatty acid (SCFA) profile. Previous studies have shown that breast-fed infants produce mainly acetate and lactate whereas in formula-fed infants, acetate, propionate and n-butyrate predominate (Edwards *et al.*, 1994). It is thought that this difference in faecal flora and faecal SCFA may be one reason for an increased incidence of enteropathic organisms and infectious diarrhoea in formula-fed infants (Howie *et al.*, 1990). Early events in the bacterial colonisation of the gut may be critical in determining the health of the infant as well as establishing the colonic flora of the adult.

The main SCFA produced by colonic fermentation of carbohydrate are acetic acid, propionic acid, butyric acid and valeric acid. The fermentation of protein gives rise to the branch-chain fatty acids, iso-butyric acid and iso-valeric acid (Macfarlane *et al.*, 1986). In addition lactate and succinate are important intermediates that may be fermented further or excreted in faeces. The individual SCFA are produced by different bacteria and not all are produced by each species (Macy and Probst, 1979; Madelstam *et al.*, 1982). The major bacteria genera which degrade polysaccharides in the colon and their respective fermentation products are shown in Table 5.1.

**Table 5.1 SCFA produced by various bacterial genera after fermentation of carbohydrate**

<b>Bacteria Genera</b>	<b>SCFA</b>
Bacteroides	acetate, propionate, succinate
Eubacterium	acetate, butyrate, lactate
Bifidobacterium	acetate, lactate
Lactobacillus	acetate, lactate
Clostridium	acetate, butyrate, propionate, lactate
Streptococci	acetate, lactate
Ruminococcus	acetate, lactate, succinate

As the microflora of the infant gut develops in the first year of life before and after weaning it is important to measure SCFA production at different ages. Previous studies of the development of the infant microflora have concentrated on the populations of bacteria present or have measured SCFA by semi-quantitative methods, reporting on the numbers of infants with a particular SCFA rather than absolute concentrations (Bullen *et al.*, 1976). Some studies have measured faecal SCFA of infants in Denmark (Rasmussen *et al.*, 1988), Sweden (Midtvedt and Midtvedt, 1992), Estonia (Siguur *et al.*, 1993) and the USA (Lifschitz *et al.*, 1990). Neonatal feeding practices differ in each of these countries. In the UK, initial rates of breast-feeding are much lower. It is not uncommon for infants to be formula-fed from birth or to receive breast-milk for a short time period before being transferred to formula-feeds. In the studies in Sweden, Denmark and Estonia most infants are breast-fed or receive some breast milk and glucose supplements before formula-milk is substituted. These studies may be of limited relevance to the UK where large numbers of infants never receive human milk.

Most of the studies have been cross-sectional or for short periods with the exception of the Swedish study. If we wish to investigate how the bacterial flora and hence the faecal SCFA change over time, a longitudinal study needs to be carried out. The study in Sweden indicated that by the age of about two years the faecal flora and the faecal SCFA profile is similar in all infants (Midtvedt and Midtvedt, 1992). This means that breast-fed infants undergo a substantial change

in their flora in the first two years of life. As formula-fed infants have a flora more like that of adults, the flora will change to a lesser extent. There have been no studies on infants that are mixed fed.

It is unclear when these changes in faecal flora of breast-fed and formula-fed infants occur but it is thought that weaning may be a critical stage. At weaning the infant is introduced to new substrates and a range of unabsorbable carbohydrate which may effect the faecal flora. If infants are presented with a substrate which they cannot ferment, it will pass through the colon unmetabolised and may cause greater stool output. Inefficient fermentation of unabsorbed carbohydrate may result in a greater tendency to diarrhoea and previous studies have shown that breast-fed infants are more susceptible to gastrointestinal infections during weaning (Gordon, 1971).

The main aim of this chapter was to examine the longitudinal development of faecal SCFA in three different infant feeding groups, breast-fed, formula-fed and mixed fed. The differences between the three feeding groups were also investigated to confirm differences in faecal SCFA profiles of breast-fed and formula-fed infants that had been shown in previous studies at pre-weaning, and to determine whether these profiles change during weaning. As a large number of mothers feed both breast and formula-milk prior to weaning we wanted to test the hypothesis that mixed fed infants changed from having a profile of a breast-fed infant to that of a formula-fed infant.

## **5.2 METHODS**

### **5.2.1 Subjects**

Three infant feeding groups were studied, breast-fed ( $n = 43$ ), formula-fed ( $n = 26$ ) and mixed fed ( $n = 19$ ) from birth through to nine months and at eighteen months. The mixed-fed group was receiving a range in quantity of formula-milk from one bottle in combination with breast-milk to complete transfer to formula

feeding after a period of breast-feeding. Four development stages were investigated in each infant, 2 months (pre-weaning), four weeks after first giving any solid food (early weaning), 9 months (late weaning) and 18 months (very late weaning).

A faecal sample was obtained at each development stage from each infant and this was weighed, frozen and freeze-dried. The freeze-dried sample was re-weighed to calculate percentage water. The dried sample was assayed by gas liquid chromatography to measure faecal SCFA and lactate to see how the profile of faecal SCFA changed with progression through weaning. Methods for sample collection and faecal short chain fatty acid assays are as per Chapter 3.

### **5.2.2 Definitions of feeding method**

Infants were classified as breast-fed if they had been exclusively fed on breast milk until the introduction of solid foods. Before weaning the only addition they may have had to breast-milk was water. The formula-fed group had been exclusively fed a modern infant formula from birth. Infants were considered mixed fed if they had been breast-fed and then transferred completely to formula milk or if they received a mixture of breast and formula milk. The characteristics of the mixed feeding group are shown in Chapter 4, Table 4.2.

Early weaning was defined as four weeks after giving solid food. This was thought to be an appropriate time when solid feeding had been established to an extent that the gut microflora may be affected by the introduction of the new substrates.

### **5.2.3 Statistics**

As there were three feeding groups and four development stages the amount of data was considerable. In addition the data was expressed as per g dry weight of faeces and per g wet weight of faeces. The wet weight value takes into account

the percentage water in the faeces of individual infants. After several discussions with several statisticians the following treatments were agreed. The data were not normally distributed. As there were four developmental stages to be followed it was considered that longitudinal data were best compared by the one-sample Wilcoxon test. However, it was suggested that as few as possible Wilcoxon tests should be made on the data as a large amount of tests would increase the possibility of differences being significant by chance. Consequently total SCFA and proportions of SCFA are tested for significance by Wilcoxon one sample test. As butyrate is considered to be the most important SCFA for colonic health (Chapter 1), it was also analysed. Because of the overall number of statistical tests this involves, more emphasis is put on statistical significance of  $p < 0.01$  rather than  $p < 0.05$ .

For comparisons between the three feeding groups a non-parametric test was used as the data was not normally distributed. Kruskal-Wallis was used in the first instance, this takes account of significant differences being found by chance. If when tested by Kruskal-Wallis there was a significant difference this was considered to be a true difference and each group (breast, formula and mixed fed) could then be compared to each other.

## **5.3 RESULTS**

In this section the longitudinal changes in each feeding group will be examined first and then differences between feeding groups will be considered.

### **5.3.1 Breast-Fed Infants**

#### **5.3.1.1 Percentage water in faecal samples**

No differences were seen in the percentage of water in the faecal samples through any of the development stages (Table 5.2). There was a great variability between infants but this was similar at each age.



**Table 5.2 Percentage water in faecal samples of infants exclusively breast-fed until weaning (n = 43)**

	Breast-fed	
	Median	Range
Pre-weaning (n = 43)	75.1	50.0 -91.0
Early weaning (n = 43)	78.5	51.6 -98.3
Late weaning (n = 43)	77.1	61.8 -84.9
Very late weaning (n = 28)	73.1	49.2 -83.3

### 5.3.1.2 Faecal SFCA of Breast-Fed Infants

The total amounts of SCFA and lactate produced by the breast-fed infants (Table 5.3) increased after weaning ( $p < 0.001$ ). At early and late weaning this difference was most marked with total SCFA and lactate peaking then dropping significantly at very late weaning ( $p < 0.05$ ) but they were still significantly higher than pre-weaning values ( $p < 0.01$ ), Table 5.3.

**Table 5.3 Total SCFA and lactate ( $\mu$ moles/g dry weight) in faeces of infants exclusively breast-fed until weaning**

	Total SCFA and lactate ( $\mu$ moles/g dry weight)	
	Median	Range
Pre-weaning (n = 43)	312.0	132.7 - 694.8
Early weaning (n = 43)	486.3***	187.8 - 1723.4
Late weaning (n = 43)	459.2***	197.9 - 876.3
Very late weaning (n = 28)	392.8***†	244.2 - 886.0

\*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  compared with pre-weaning, †  $p < 0.05$  compared with late weaning by Wilcoxon.

Total faecal SCFA per g wet weight were also analysed, this takes into account the percentage water in the faeces of an individual. As the percentage water varies greatly between individuals the total amount of faecal SCFA of each infant will differ if this is taken into account. However, the concentration / g wet weight

is more relevant as this is what the microflora and pathogenic bacteria will be exposed to. When the total faecal SCFA were expressed as per g wet weight a similar pattern was observed. The total SCFA including lactate was significantly lower at pre-weaning than at the other development stages ( $p<0.01$ ) (Table 5.4). Again there was an increase in SCFA in early and late weaning that after peaking, tailed off in very late weaning, but this was still significantly higher than pre-weaning ( $p<0.05$ ).

**Table 5.4**                      **Total SCFA including lactate per gramme wet weight ( $\mu\text{mol/ml}$ ) in faeces of infants exclusively breast-fed until weaning**

	Total SCFA including lactate wet weight ( $\mu\text{mol/ml}$ )	
	Median	Range
Pre-weaning (n = 43)	68.0	23.9 - 165.8
Early weaning (n = 43)	108.7**	7.2 - 296.6
Late weaning (n = 43)	110.7**	33.0 - 237.0
Very late weaning (n = 28)	102.6*	66.2 - 255.9

\* $p<0.05$ , \*\* $p<0.01$  compared with pre-weaning by Wilcoxon.

Breast-fed infants characteristically produce predominantly acetate and lactate (Edwards *et al.*, 1994). Previous studies (Rasmussen *et al.*, 1988; Midtvedt and Midtvedt, 1992; Siguur *et al.*, 1993) have not included lactate concentration so to allow comparisons with previous studies the SCFA were additionally statistically compared without lactate. Total SCFA excluding lactate increased after weaning ( $p<0.001$ ). At very late weaning the amount of SCFA excluding lactate had decreased but was still significantly higher than pre-weaning values ( $p<0.01$ ). The difference seen when lactate was included with the SCFA, between late and very late weaning, was no longer present (Table 5.5).

Results for the wet weight total SCFA without lactate are shown in Table 5.5 and these replicate the results for SCFA including lactate. The pre-weaning total was significantly lower than the other development stages ( $p < 0.01$ ,  $p < 0.001$ ).

**Table 5.5** **Total SCFA without lactate (dry weight) in faeces of infants exclusively breast-fed until weaning (n=43).**

	Dry Weight ( $\mu\text{mol/g}$ )		Wet Weight ( $\mu\text{mol/ml}$ )	
	Median	Range	Median	Range
PW	262.6	61.9 - 662.8	61.1	16.5 - 160.1
EW	421.1***	67.2 - 734.0	81.7**	7.2 - 193.7
LW	447.5***	197.9 - 800.9	103.5***	33.0 - 213.5
VLW (n=28)	392.2**	206.7 - 886.0	102.6**	64.0 - 255.9

\*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared with pre-weaning by Wilcoxon.

PW = Pre-weaning, EW = Early weaning, LW = Late weaning, VLW = Very late weaning

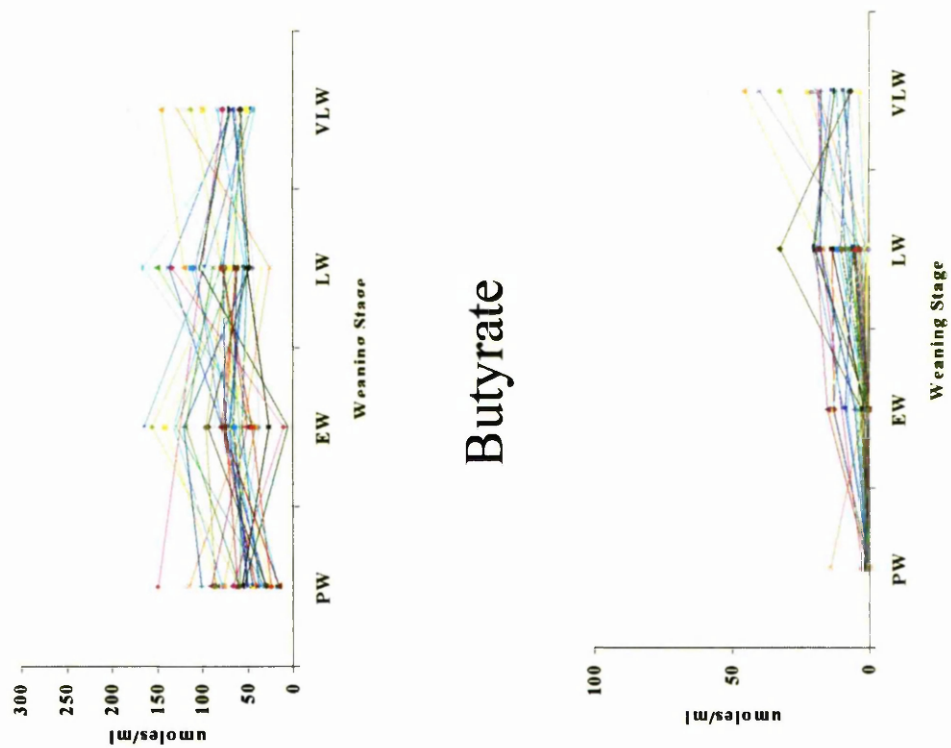
### 5.3.1.3 Proportions of Individual SCFA

The percentage of acetate and lactate tended to fall between pre-weaning and very late weaning (Table 5.6). This was statistically significant only with lactate ( $p < 0.001$ ). Conversely propionate and n-butyrate increased significantly ( $p < 0.001$ ).

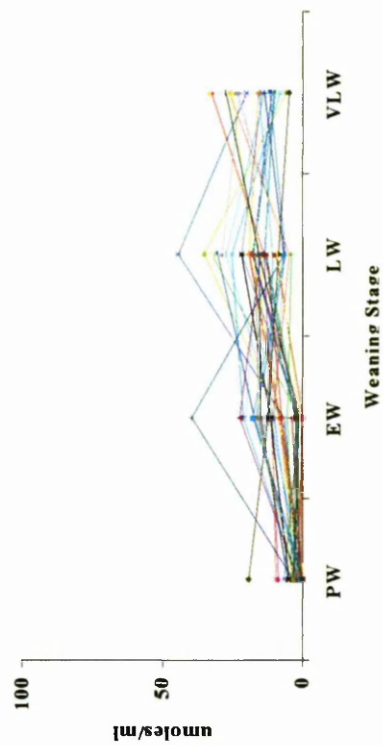
The individual data for the total SCFA and lactate (percentages and wet weight) were also plotted (Figure 5.1, 5.2). Individual infants were seen to vary considerably over the four development stages.

Figure 5.1 Total SCFA of breast-fed infants wet weight

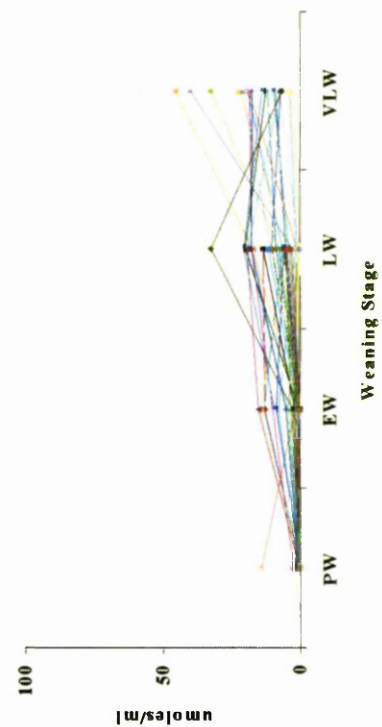
## Acetate



## Propionate



## Butyrate



## Lactate

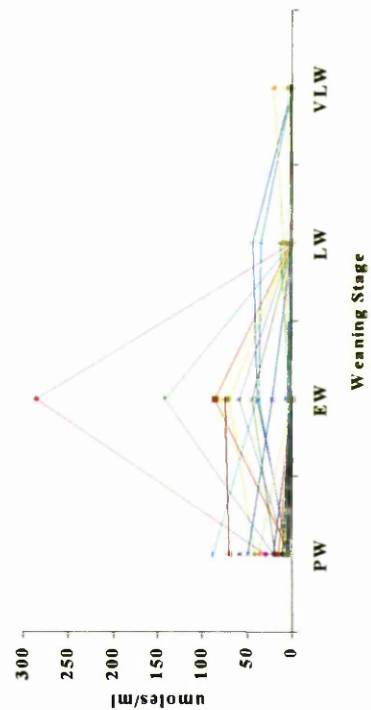
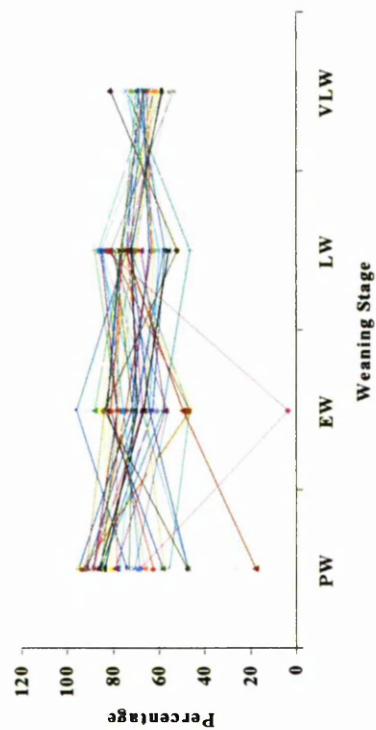
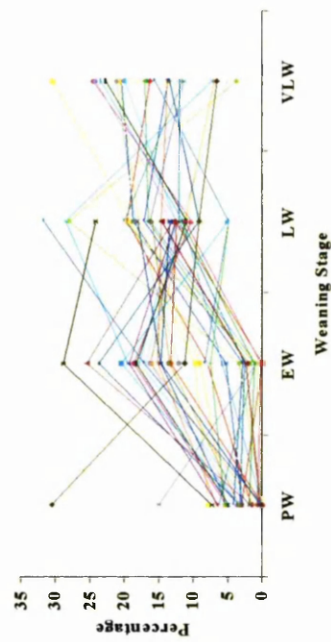


Figure 5.2 Total SCFA of breast-fed infants percentages

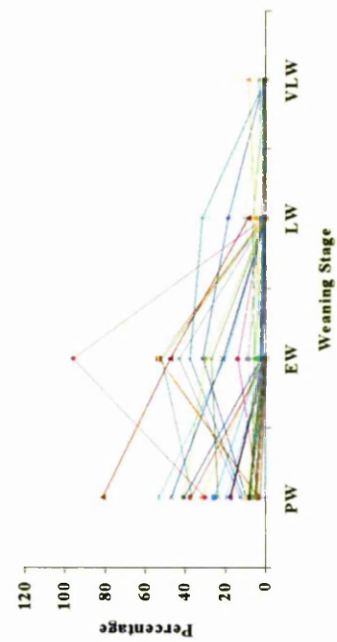
## Percentage acetate



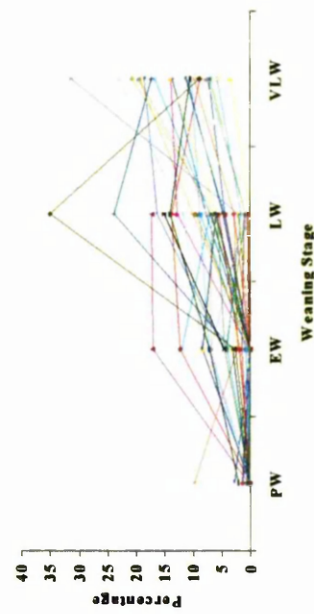
## Percentage propionate



## Percentage lactate



## Percentage butyrate



**Table 5.6 Median values for percentage of individual SCFA in faeces of infants exclusively breast-fed until weaning (n = 43).**

	Breast-fed			
	% Acetate	% Propionate	% N-Butyrate	% Lactate
PW	80.0	3.2	0	11.1
EW	70.5	11.2 <sup>***</sup>	1.5 <sup>**</sup>	2.5
LW	74.1	13.1 <sup>***†</sup>	6.1 <sup>****††</sup>	0.0 <sup>****†††</sup>
VLW(n=28)	66.1 <sup>†</sup>	15.9 <sup>**†</sup>	11.8 <sup>****†††‡</sup>	0.0 <sup>****†</sup>

\*\* p<0.01, \*\*\* p<0.001 compared with pre-weaning, † p<0.05, †† p<0.01, ††† p<0.001 compared with early weaning, ‡‡ p<0.01 compared with late weaning by Wilcoxon  
PW = Pre-weaning, EW = Early weaning, LW = Late weaning, VLW = Very late weaning

Butyrate is considered to be the most important SCFA for colonic health (Chapter 1). Very little was seen in young neonates. To examine this in more detail the amount of n-butyrate produced was expressed per gramme wet and dry weight and statistically compared. Pre-weaning n-butyrate (by both methods) was significantly lower than all other development stages (p<0.01) (Table 5.7). Butyrate (/g dry weight, / g wet weight and percentages) was significantly lower at early weaning than both late and very late weaning (p<0.001). There was also a significant increase between late and very late weaning (p<0.05).

**Table 5.7 Median values for n-butyrate in faeces of infants exclusively breast-fed until weaning (n = 43).**

	n-Butyrate (μmol)		
	/g dry faeces	/g wet faeces	%
Pre-weaning	0	0	0
Early weaning	5.5 <sup>**</sup>	0.8 <sup>**</sup>	1.5 <sup>**</sup>
Late weaning	27.1 <sup>****†††</sup>	7.6 <sup>****†††</sup>	6.1 <sup>****†††</sup>
Very late weaning (n=28)	51.2 <sup>****†††‡</sup>	13.8 <sup>****†††‡</sup>	11.8 <sup>****†††‡</sup>

\*p<0.05, \*\*p<0.01, \*\*\*p<0.001 compared with pre-weaning, †††p<0.001 compared with early weaning, ‡p<0.05, ‡‡p<0.01 compared with late weaning by Wilcoxon.

### 5.3.2 Formula-Fed Infants

#### 5.3.2.1 Percentage Water in Faecal Samples

There were no significant differences in faecal water between any of the development stages (Table 5.8). As with breast-fed infants, there was considerable variability between individual infants.

**Table 5.8 Percentage water in faecal samples from exclusively formula-fed infants (n = 26).**

	Formula-fed	
	Median	Range
Pre-weaning	73.4	64.0-82.2
Early weaning	73.1	67.8-85.7
Late weaning	73.7	43.5-80.8
Very late weaning (n=16)	75.2	51.8-85.4

#### 5.3.2.2 Faecal SFCA of Formula-Fed Infants

There were no significant differences in total SCFA and lactate produced by formula-fed infants between any development stage (Table 5.9). Similarly no differences were seen in total SCFA and lactate wet weight over time.

**Table 5.9 Total SCFA including lactate in faeces of infants exclusively formula-fed until weaning (n=26)**

	SCFA Dry Weight ( $\mu$ moles/g)		SCFA Wet Weight ( $\mu$ mol/ml)	
	Median	Range	Median	Range
Pre-weaning	361.5	163.1 -601.6	86.4	41.8 - 147.7
Early weaning	383.8	213.0 -870.8	103.4	39.0 - 239.0
Late weaning	402.9	205.8 -967.1	101.3	40.1 - 353.1
Very late weaning (n=16)	448.9	129.7 -973.6	118.7	29.8 - 238.4

When the values for total SFCA without lactate (wet and dry) were compared the only significant difference seen was that the total SCFA (wet weight) for the pre-weaning group was significantly lower than that of the late weaning group ( $p<0.05$ ), Table 5.10. Formula-fed infants produced lactate less often (although one or two individuals may produce it in large quantities) and the difference seen may be due to high lactate production in the late weaning group by some individual infants.

**Table 5.10** Total SCFA without lactate /g (wet and dry weight) in faeces of infants exclusively formula-fed until weaning (n=26)

	SCFA			
	Dry weight ( $\mu$ moles/g)		Wet weight (mmol/ml)	
	Median	Range	Median	Range
Pre-weaning	361.5	156.9- 578.4	86.3	41.8 - 146.1
Early weaning	378.3	213.0- 868.4	101.8	39.0 - 238.4
Late weaning	399.1	205.8- 964.3	100.2*	40.1 - 351.5
Very late	447.8	129.7- 908.3	114.7	29.8 - 222.4

\* $p<0.05$  compared with pre-weaning by Wilcoxon.

### 5.3.2.3 Proportions of Individual SCFA

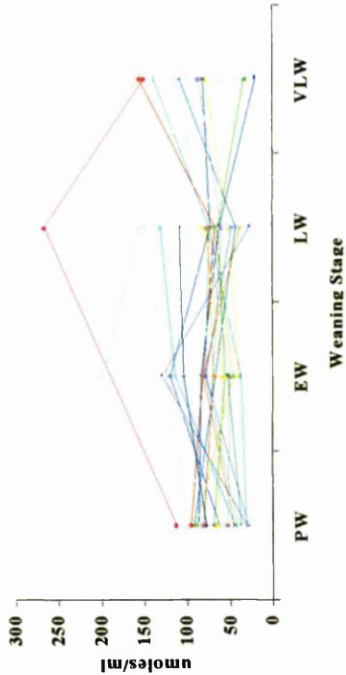
Very few statistical differences were seen between the percentage of each SCFA through the development stages (Table 5.11). At late and very late weaning there was significantly more n-butyrate in faeces than at pre-weaning ( $p<0.05$ ). Butyrate and lactate were significantly higher at very late weaning than at early weaning ( $p<0.05$ ). Very little lactate was produced by the formula-fed infants at any development stage.

SCFA values (percentages and / g wet weight) of individual infants were plotted against development are shown in figures 5.3, 5.4.

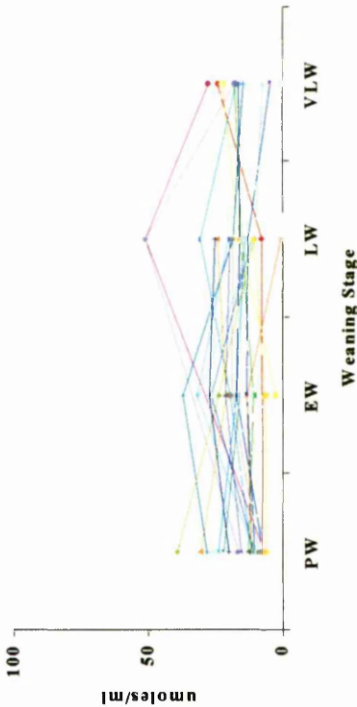


Figure 5.3 Total SCFA of formula-fed infants wet weight

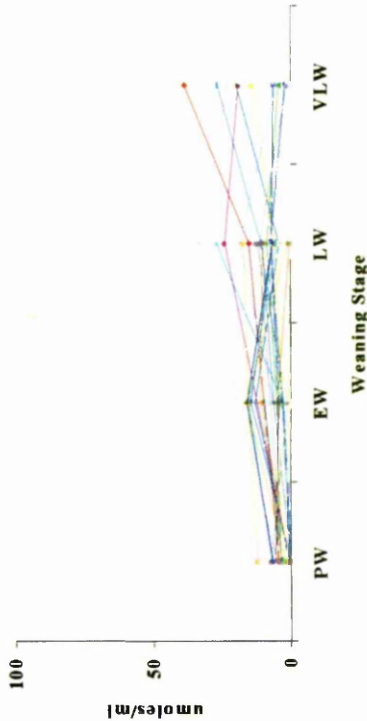
Acetate



Propionate



Butyrate



Lactate

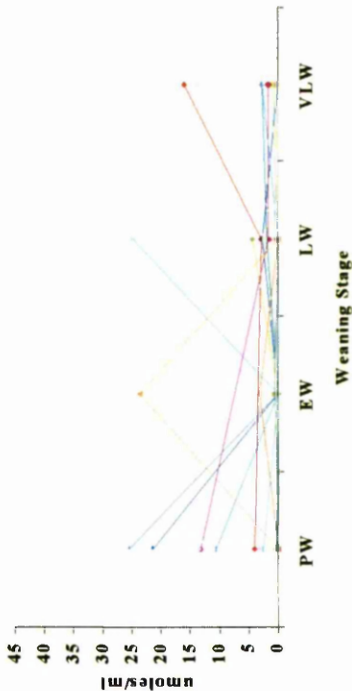
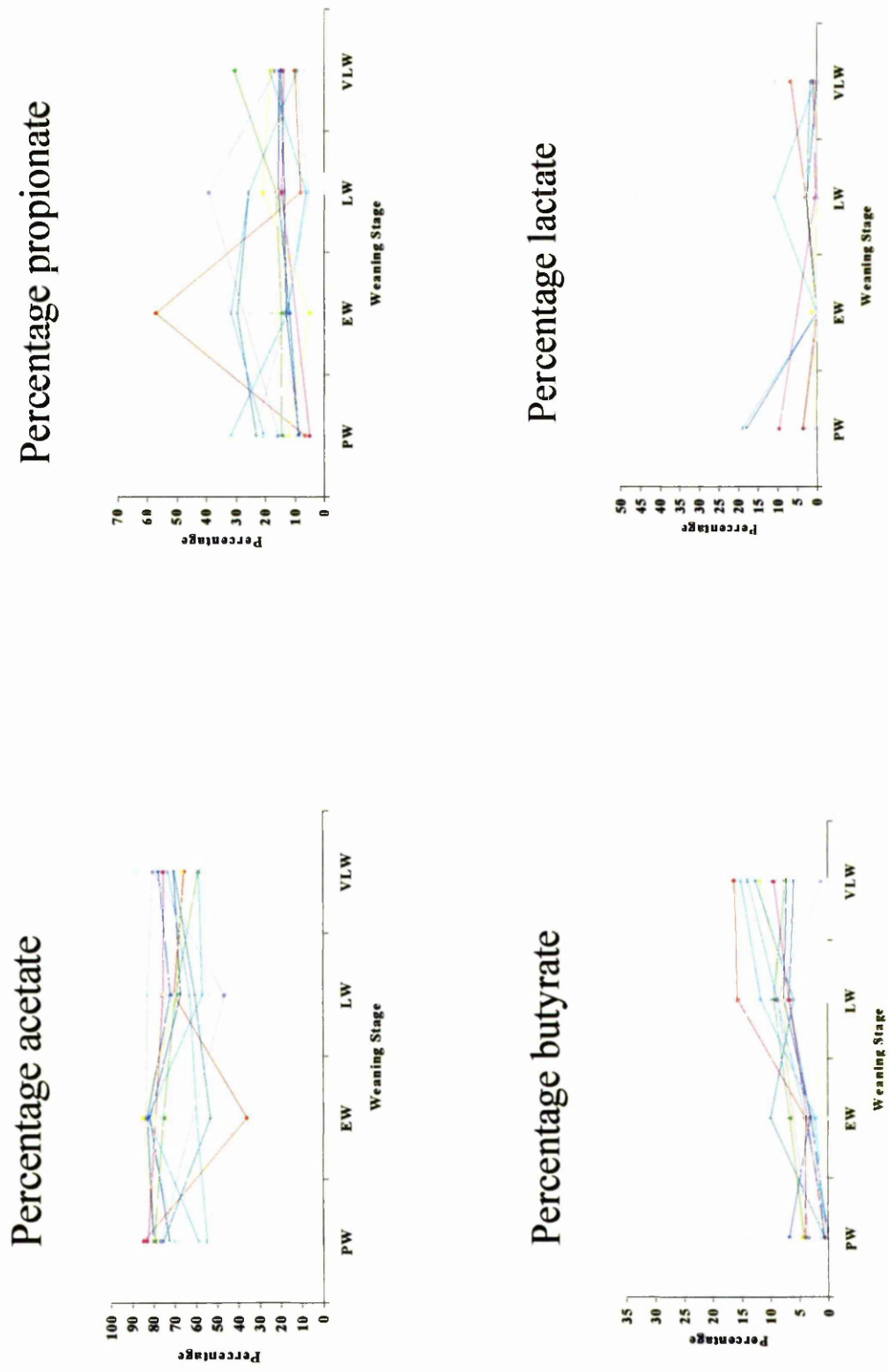


Figure 5.4 Total SCFA of formula-fed infants percentages



**Table 5.11 Median values for percentage of individual SCFA in faeces of infants exclusively formula-fed until weaning (n=26)**

	Formula-fed			
	Acetate	Propionate	n-Butyrate	Lactate
Pre-weaning	71.8	15.4	3.7	0
Early weaning	72.5	16.5	4.9	0
Late weaning	68.9	16.2	7.7*	0
Very late weaning (n=16)	71.7	12.0	8.4*†	0.7†

\*p<0.05 compared with pre-weaning, † p<0.05 compared with early weaning by Wilcoxon.

Table 5.12 shows n-butyrate concentration expressed per gramme as wet, dry weight and percentage of total SCFA. In the formula-fed group, there were measurable amounts of n-butyrate at all ages. When compared as a value of dry faecal weight (p<0.01, P<0.001) and of wet faecal weight matter (p<0.05, p<0.01) butyrate at pre-weaning was significantly lower than at early and late weaning. Butyrate increased between early weaning and very late weaning when results were expressed as dry weight (p<0.001). When expressed as dry weight and percentage there was significantly more n-butyrate at very late weaning than at late weaning (p<0.05).

**Table 5.12 Median values for n-butyrate in faeces of infants exclusively formula-fed until weaning (n=26).**

	/mg dry faeces	/mg wet faeces	%
Pre-weaning	11.6	3.6	3.7
Early weaning	19.2**	5.6*	4.9
Late weaning	29.5***†††	8.6**	7.7*
Very late weaning	32.2***††††	6.2	8.4*†

\*p<0.05, \*\*p<0.01, \*\*\* p< 0.001 compared with pre-weaning , †p<0.05, †††p<0.05 compared with early weaning, †† p<0.05 compared with late weaning by Wilcoxon.

### 5.3.3 Mixed Fed Infants

#### 5.3.3.1 Percentage Water in Faecal Samples

A significant difference in faecal water was seen only between late weaning and very late weaning (Table 5.13). At very late weaning the percentage water was significantly lower than at late weaning ( $p < 0.05$ ). This difference, however, may be due to chance as so many statistical tests were carried out. A large variation was seen in percentage water between individual infants.

**Table 5.13** Percentage water in faecal samples from mixed fed infants (n = 19)

	Mixed-fed	
	Median	Range
Pre-weaning	72.4	58.7 - 78.2
Early weaning	71.2	49.2 - 99.0
Late weaning	74.5	55.2 - 84.9
Very late weaning	68.9*	49.2 - 84.0

\*  $p < 0.05$  compared with late weaning by Wilcoxon.

#### 5.3.2.2 Faecal SFCA of Mixed Fed Infants

The total amount of SCFA and lactate produced by the mixed fed infants were significantly lower at pre-weaning than in the other age groups ( $p < 0.01$ ,  $p < 0.05$ ). There was a trend for increased total SCFA including lactate from pre-weaning to very late weaning (Table 5.14).

**Table 5.14** Total SCFA including lactate ( $\mu$ moles/g dry weight) in faeces of mixed fed infants (n=19).

	Mixed-fed	
	Median	Range
Pre-weaning	256.7	132.9 - 618.4
Early weaning	430.6*	234.7 - 615.8
Late weaning	425.5**	198.4 - 841.1
Very late weaning	465.3**	187.6 - 761.7

\*  $p < 0.05$ , \*\*  $p < 0.01$  compared with pre-weaning by Wilcoxon

Similarly, when the faecal values were expressed as / g wet weight, for mixed fed infants values were significantly higher in all groups than at pre-weaning (Table 5.15,  $p<0.01$  and  $p<0.05$ ). The value at very late weaning was significantly higher than at late weaning ( $p<0.05$ ).

**Table 5.15**      **Total SCFA including lactate wet weight (mmol/ml) in faeces of mixed fed infants (n=19)**

	Mixed-fed			
	Median	Range		
Pre-weaning	83.8	40.9	-	144.6
Early weaning	139.4*	4.2	-	222.3
Late weaning	112.9**	52.0	-	179.2
Very late weaning	138.9**†	31.3	-	260.8

\*  $p<0.05$ , \*\*  $p<0.01$  compared with pre-weaning, † compared to late weaning by Wilcoxon

When total SCFA results were compared without lactate the pre-weaning group had significantly lower values than all other groups for data expressed as wet and dry faecal weight  $p<0.01$ , Table 5.16).

**Table 5.16**      **Total SCFA without lactate in faeces of mixed fed infants (n=19)**

	Mixed-fed			
	Dry weight (μmoles/g)		Wet weight (mmol/ml)	
	Median	Range	Median	Range
Pre-weaning	254.8	120.5-560.3	83.8	40.9 - 138.8
Early weaning	426.9**	234.7-615.8	138.8**	4.2 - 222.3
Late weaning	425.5**	198.4-841.1	112.9**	52.0 - 178.0
Very late weaning	465.3**	187.6-714.7	135.4**	31.3 - 250.5

\*\* $p<0.01$  compared with pre-weaning by Wilcoxon.

### 5.3.3.3 Proportions of Individual SCFA

The percentage of individual SCFA at each development stage are shown in Table 5.17. Butyrate increased between pre-weaning and late weaning ( $p<0.01$ ). Very little lactate was produced.

Total SCFA and lactate for individual infants (percentages and / g wet weight) were also plotted so that trends can be seen. Individual infants varied considerably over the four development stages (Figures 5.5, 5.6).

**Table 5.17** Median values for percentage of individual SCFA in mixed fed infants (n = 19)

	Mixed fed			
	Acetate	Propionate	n-Butyrate	Lactate
Pre-weaning	72.8	17.6	1.4	0
Early weaning	70.7	18.3	5.7**	0.6
Late weaning	71.5	15.5	7.0****	0 <sup>†</sup>
Very late weaning	61.5***††	15.3	11.7***††	0

\*\*  $p<0.01$ , \*\*\*  $p<0.001$  compared with pre-weaning, †  $p<0.05$ , ††  $p<0.01$  compared with early weaning, ‡  $p<0.05$ , ‡‡‡  $p<0.001$  compared to late weaning by Wilcoxon

When n-butyrate was compared as a value of dry faecal weight, wet faecal weight and percentages it was found that pre-weaning butyrate was significantly lower than at all other development stages ( $p<0.01$ ,  $p<0.001$ ). Butyrate increased also between early weaning and both late and very late weaning ( $p<0.01$ ). There was also a significant increase between late and very late weaning ( $p<0.01$ ) (Table 5.18).

Figure 5.5 Total SCFA of mixed fed infants wet weight

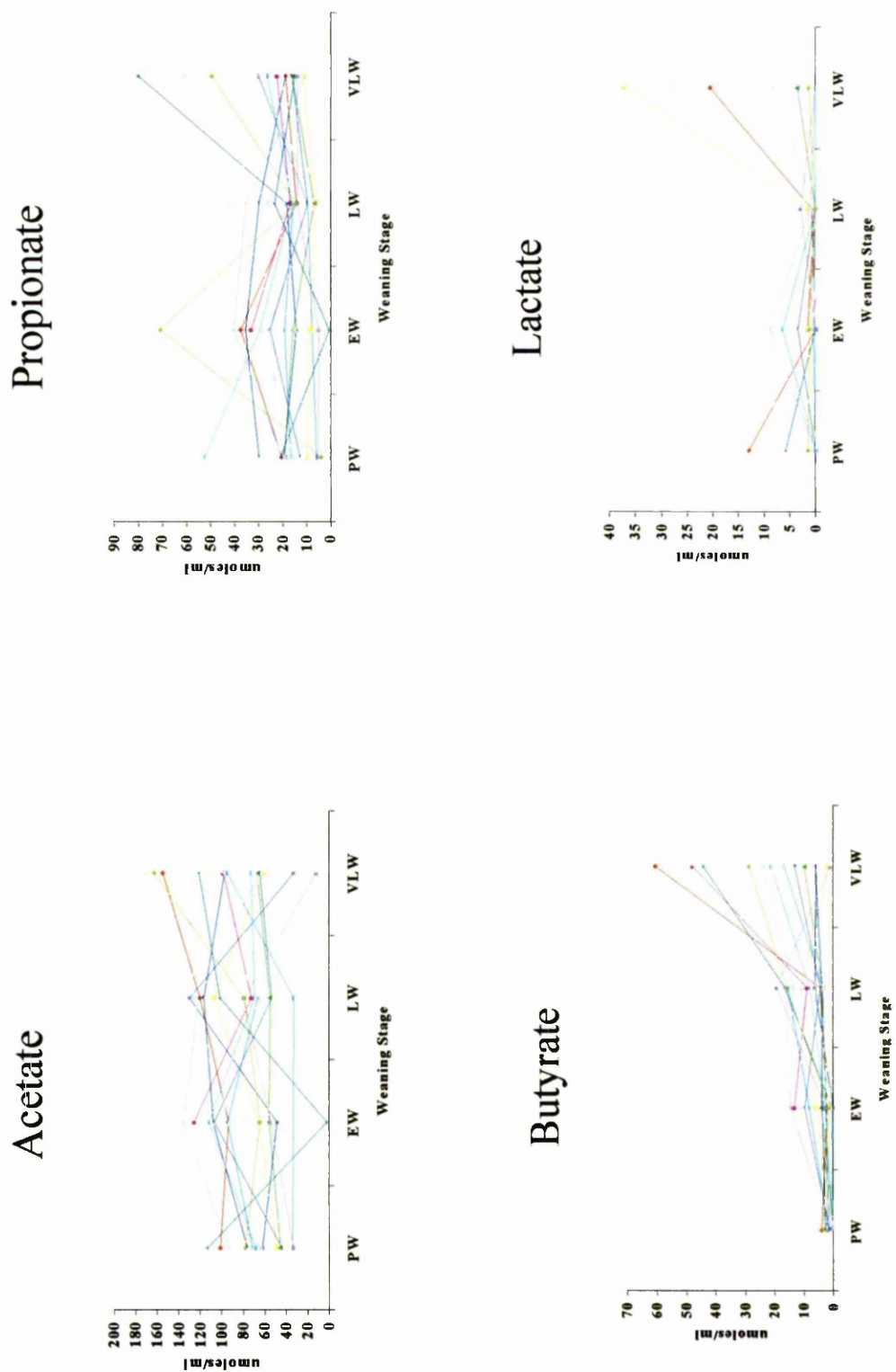
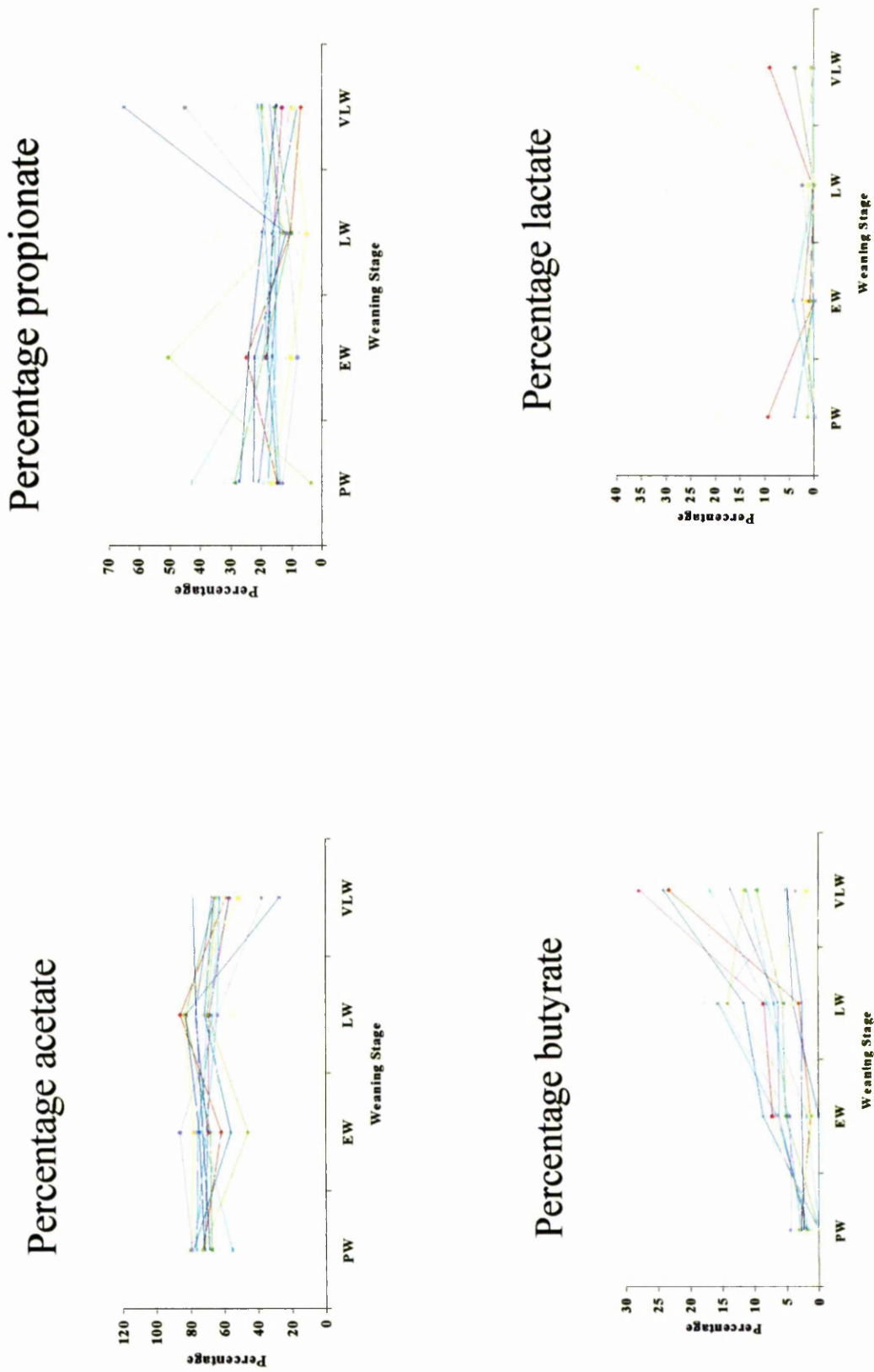


Figure 5.6 Total SCFA of mixed fed infants percentages





**Table 5.18 Median values for n-butyrate in faeces of mixed fed infants (n=19)**

	/mg dry faeces	/mg wet faeces	%
Pre-weaning	3.2	0.9	1.4
Early weaning	20.5**	4.1**	5.7**
Late weaning	25.8****†	8.1***	7.0****†
Very late weaning	63.7****††	19.2****††	11.7****†

\*\*p<0.01, \*\*\*p<0.001 compared with pre-weaning, ††p<0.01 compared with early weaning, †p<0.05 ††p<0.01 compared with late weaning by Wilcoxon.

## 5.4 COMPARISON OF BREAST-FED, FORMULA-FED AND MIXED FED INFANTS

### 5.4.1 Percentage water in faecal samples

Breast-fed infants had higher percentage water in faeces than the other two groups at early weaning (p<0.05, p<0.01 respectively) and also had higher percentage water in faeces compared with the formula fed infants at late weaning (p<0.01), Table 5.19.

**Table 5.19 Percentage water in faecal samples of three different infant feeding groups**

	Breast-fed (n = 43)		Formula-fed (n = 26)		Mixed fed (n = 19)	
	Median	Range	Median	Range	Median	Range
PW	75.1	50.0 -91.0	73.4	64.0 - 82.2	72.4	58.7 - 78.2
EW	78.5	51.6 -98.3	73.1*	67.8 - 85.7	71.2**	49.2 - 99.0
LW	77.1	61.8 -84.9	73.7**	43.5 - 80.8	74.5	55.2 - 84.9
VLW	73.1	49.2 -83.3	75.2	51.8 - 85.4	68.9	49.2 - 84.0

p<0.05, \*\*p<0.01 compared with breast-fed by Kruskal-Wallis and Mann-Whitney.

PW = Pre-weaning, EW = Early weaning, LW = Late weaning, VLW = Very late weaning

#### 5.4.4.2 Faecal SCFA of Three Different Infant Feeding Groups

There were no significant differences between the three feeding groups in total SCFA including lactate (Table 5.20). However at pre-weaning and the early weaning period there were significant differences between breast-fed and both other groups for propionate (p<0.001 at pre-weaning and p<0.01 at early weaning) and n-butyrate (p<0.05), breast-fed infants had less propionate and n-butyrate.

Whilst there was significantly higher lactate in breast-fed infants than the formula or mixed fed infants at pre-weaning ( $p<0.001$ ), this difference had disappeared by early weaning between breast-fed and mixed fed infants. At early weaning there was also significantly more acetate in the breast-fed infants than both formula-fed and mixed fed infants ( $p<0.05$ ). Significantly more n-butyrate was produced by the formula-fed infants compared with the mixed fed group at pre-weaning ( $p<0.01$ ) and significantly less lactate produced by formula-fed infants than mixed fed infants ( $p<0.05$ ) at early weaning. By the time of late and very late weaning there were no significant differences between any of the SCFA between any groups.

**Table 5.20** Total SCFA including lactate  $\mu\text{moles/g}$  dry weight in the different infant feeding groups

	Breast-fed (n = 43)		Formula-fed (n = 26)		Mixed fed (n = 19)	
	Median	Range	Median	Range	Median	Range
PW	312.0	132.7- 694.8	361.5	163.1-601.6	256.7	132.9-618.4
EW	486.3	187.8-1723.4	383.8	213.0-870.8	430.6	234.7-615.8
LW	459.2	197.9- 876.3	402.9	205.8-967.1	425.5	198.4-841.1
VLW	392.8	244.2- 886.0	448.9	129.7-973.6	465.3	187.6-761.7

\* $p<0.05$  compared with breast-fed by Kruskal-Wallis and Mann-Whitney.

PW = Pre-weaning, EW = Early weaning, LW = Late weaning, VLW = Very late weaning

When the data was expressed for wet faecal matter there were no differences in feeding groups at any of the development stages for total SCFA including lactate (Table 5.21). There were, however, differences in individual SCFA. Significantly less propionate ( $p<0.001$ ) and n-butyrate ( $p<0.05$ ) was produced by the breast-fed and than with both other feeding groups at pre and early weaning. Significantly more lactate was produced at pre-weaning by breast-fed infants than by other groups ( $p<0.001$ ). By early weaning this difference remained only between breast-fed and formula-fed ( $p<0.01$ ). Formula-fed and mixed fed group differed

only with n-butyrate at pre-weaning, where the formula-fed infants produced significantly less ( $p<0.01$ ).

**Table 5.21 Total SCFA including lactate wet weight (mmol/ml) in the different infant feeding groups.**

	Breast-fed (n = 43)		Formula-fed (n = 26)		Mixed fed (n = 19)	
	Median	Range	Median	Range	Median	Range
PW	68.0	23.9 -165.8	86.4	41.84-147.7	83.8	40.9 -144.6
EW	108.7	7.2 -296.6	103.4	39.0 -239.0	139.4	4.2 -222.3
LW	110.7	33.0 -237.0	101.3	40.1 -353.1	112.9	52.0 -179.2
VLW	102.6	66.2 -255.9	118.7	29.8 -238.4	138.9	31.3 -260.8

PW = Pre-weaning, EW = Early weaning, LW = Late weaning, VLW = Very late weaning

The only significant difference seen when comparing total SCFA without lactate were between breast-fed and formula-fed infants at pre-weaning ( $p<0.01$ ), Formula-fed infants produce more SCFA when the data were expressed as wet or dry weight (Table 5.22, 5.23).

**Table 5.22 Total SCFA without lactate dry weight ( $\mu$ moles/g)**

	Breast-fed (n = 43)		Formula-fed (n = 26)		Mixed fed (n = 19)	
	Median	Range	Median	Range	Median	Range
PW	262.6	61.9 - 662.8	361.5**	156.9-578.4	254.8	120.5-560.3
EW	421.1	67.2 - 734.0	378.3	213.0-868.4	426.9	234.7-615.8
LW	447.5	197.9 - 800.9	399.1	205.8-964.3	425.5	198.4-841.1
VLW	392.2	206.7 - 886.0	447.8	129.7-908.3	465.3	187.6-714.7

\*\*  $p<0.01$  compared with breast-fed by Kruskal-Wallis and Mann-Whitney PW = Pre-weaning, EW = Early weaning, LW = Late weaning, VLW = Very late weaning

**Table 5.23 Total SCFA without lactate wet weight (mmol/ml) in the different infant feeding groups**

	Breast-fed (n = 43)		Formula-fed (n = 26)		Mixed fed (n = 19)	
	Median	Range	Median	Range	Median	Range
PW	61.1	16.5 - 160.1	86.3**	41.8 -146.1	83.8	40.9 -138.8
EW	81.7	7.2 - 193.7	101.8	39.0 -238.4	138.8	4.2 -222.3
LW	103.5	33.0 - 213.5	100.2	40.1 -351.5	112.9	52.0 -178.0
VLW	102.6	64.0 - 255.9	114.7	29.8 -222.4	135.4	31.3 -250.5

\*\* p<0.01 compared with breast-fed by Kruskal-Wallis and Mann-Whitney

PW = Pre-weaning, EW = Early weaning, LW = Late weaning, VLW = Very late weaning

#### 5.4.5 Proportions of Individual SCFA

The breast-fed infants had several differences in the proportions of individual SCFA from both other feeding groups (Table 5.24). There was less propionate in breast-fed infants at pre (p<0.001) and early weaning (p<0.01). Similarly there was less n-butyrate at pre- (p<0.001 compared with formula-fed and p<0.05 compared with mixed fed) and early weaning (p<0.01 compared with formula-fed and p<0.05 compared with mixed fed). More lactate was produced by breast-fed infants at pre-weaning (p<0.001), by early weaning the difference was not as great but still present weaning (p<0.01) compared with formula-fed and p<0.05 compared with mixed fed). Formula-fed infants differed from mixed-fed infants only in the production of n-butyrate (formula-fed infants produced significantly more, p<0.05) at pre-weaning and acetate (formula-fed infants produced significantly more, p<0.0) at very late weaning. The only difference seen in the latter age groups was with the production of acetate at very late weaning, breast-fed infants had significantly less than formula-fed infants (p<0.05), and significantly more than mixed fed infants (p<0.05).

**Table 5.24 Median values of percentage of individual SCFA in the different infant feeding groups**

Breast-fed (n =43)		%		
	Acetate	Propionate	N-Butyrate	Lactate
PW	80.0	3.2	0.0	11.1
EW	70.5	11.2	1.5	2.5
LW	74.1	13.1	6.1	0.0
VLW	66.1	15.9	11.8	0.0
Formula-fed (n = 26)				
	Acetate	Propionate	N-Butyrate	Lactate
PW	71.8	15.4 <sup>***</sup>	3.7 <sup>***</sup>	0.0 <sup>***</sup>
EW	72.5	16.5 <sup>**</sup>	4.9 <sup>**</sup>	0.0 <sup>**</sup>
LW	68.9	16.2	7.7	0.0
VLW	71.7 <sup>*</sup>	12.0	8.4	0.7
Mixed fed (n = 19)				
	Acetate	Propionate	N-Butyrate	Lactate
PW	72.8	17.6 <sup>***</sup>	1.4 <sup>††</sup>	0.0 <sup>***</sup>
EW	70.7	18.3 <sup>**</sup>	5.7 <sup>*</sup>	0.6
LW	71.5	15.5	7.0	0.0
VLW	61.5 <sup>††</sup>	15.3	11.7	0.0

\*p<0.05, \*\*p<0.01, \*\*\*p<0.001 compared with breast-fed, † p<0.05, †† p<0.01 compared with formula-fed by Kruskal-Wallis and Mann-Whitney.

PW = Pre-weaning, EW = Early weaning, LW = Late weaning, VLW = Very late weaning

For data expressed as dry weight, wet weight or percentage (Table 5.26), there was significantly less n-butyrate in breast-fed than both formula-fed ( $p<0.001$  at pre and  $p<0.01$  at early weaning) and mixed fed at pre- and early weaning ( $p<0.05$ ). There was significantly less n-butyrate in mixed fed infants compared with formula-fed infants at pre-weaning ( $p<0.01$  for wet and dry weight,  $p<0.05$  for percentages) and by early weaning this difference had disappeared. There were no differences between the three feeding groups at late or very late weaning.

**Table 5.26 Median values for n-butyrate in the different infant feeding groups**

Breast-fed (n = 43)	$\mu\text{mol/ml}$		
	/mg dry faeces	/mg wet faeces	%
PW	0	0	0
EW	5.5	0.8	1.5
LW	27.1	7.6	6.1
VLW	51.2	13.8	11.8
<b>Formula-fed (n = 26)</b>			
PW	11.6 <sup>***</sup>	3.6 <sup>***</sup>	3.7 <sup>***</sup>
EW	19.2 <sup>**</sup>	5.6 <sup>**</sup>	4.9 <sup>**</sup>
LW	29.5	8.6	7.7
VLW	32.2	6.2	8.4
<b>Mixed-fed (n = 19)</b>			
PW	3.2 <sup>*††</sup>	0.9 <sup>*††</sup>	1.4 <sup>*†</sup>
EW	20.5 <sup>*</sup>	4.1 <sup>*</sup>	5.7 <sup>*</sup>
LW	25.8	8.1	7.0
VLW	63.7	19.2	11.7

\*p<0.05, \*\*p<0.01, \*\*\*p<0.001 compared to breast-fed, †p<0.05,††p<0.01 compared with formula-fed by Kruskal-Wallis and Mann-Whitney.

PW = Pre-weaning, EW = Early weaning, LW = Late weaning, VLW = Very late weaning

## 5.5 DISCUSSION

It is well established that the faecal flora of exclusively breast-fed and exclusively formula-fed infants differs (Balmer and Wharton, 1989). This difference is reflected in the faecal SCFA produced by the two groups of infants (Edwards *et al.*, 1994). This may persist until about the age of two years when the flora appears to become like that of the adult (Midtvedt and Midtvedt, 1992). This change is more noticeable in breast-fed infants than in formula-fed infants who have a faecal flora more like that of an adult to start with. Very little is known

about what happens between the pre-weaning stage and the age of two years. It is unclear what factors induce this change in microflora.

As discussed in Chapter 1, it is thought that there are two stages that are critical to the development of the flora. These stages are at birth and weaning. At birth, the colon is colonised by bacteria introduced by the mother's skin, vagina and faeces (Nolte, 1977; Tannock, 1990). Feeding practice then influences colonisation. The period of weaning has not been fully investigated but it is likely that as the infant comes into contact with new substrates the bacteria changes. During weaning, the simple flora develops until the adult microflora is established with approximately 400 different bacterial species. It is not known how quickly this colonisation occurs or what dietary influences are involved.

Prior to weaning a significant amount of dietary carbohydrate may escape digestion because of the immature activity of pancreatic and brush border enzymes (McClellan and Weaver, 1993). This dietary carbohydrate consists of mainly lactose and fructo-oligosaccharides in breast-fed infants but other carbohydrates may be present in formula milk. As weaning begins, infants are exposed for the first time to many complex carbohydrates including dietary fibre and starch. These carbohydrates may escape digestion because of lack of chewing ability and pancreatic exocrine function in these young children. These substrates will pass through to the colon where they may be anaerobically metabolised to produce SCFA, lactate and gases. Inefficient fermentation of unabsorbed carbohydrate may result in a greater tendency to diarrhoea and previous studies have shown that breast-fed infants are more susceptible to gastrointestinal infections during weaning (Gordon, 1971; Lifschitz, 1996).

Obviously in the first year of life the infant is also increasing in age and the gut and its function mature. This maturation may be independent of diet. It is impossible to separate the effects of age from those of weaning without a very large number of infants who were weaned at quite different ages or who had weaning.

In breast-fed infants total SCFA including lactate (dry weight) increased from pre-weaning ( $p < 0.001$  at early and late weaning,  $p, 0.01$  at very late weaning). Similar results were seen when data was expressed as wet weight. When lactate was not included this increase in total SCFA from pre-weaning still remained. In mixed fed infants there was also an increase in total SCFA including lactate (dry and wet weight) from pre-weaning ( $p < 0.05$ ). When data was analysed without lactate these increases became more significant. No differences were seen with formula-fed infants at any development stage for total SCFA including lactate (dry and wet weight). When lactate was not included the only difference was an increase between pre- and late weaning ( $P < 0.05$ ).

Breast-fed infants had a profile of mainly acetate and lactate at pre-weaning but at early weaning the amount of lactate decreased, although not significantly. Propionate and butyrate increased from pre-weaning to early weaning ( $p < 0.001$ ,  $0.01$  respectively). At late weaning there was a significant decrease in lactate from pre- and early weaning ( $p < 0.001$ ) and this remained at very late weaning. Propionate and butyrate continued to increase through late and very late weaning.

The mixed fed group differed in that the profile did not show any lactate at pre-weaning but mainly acetate and propionate. Butyrate increased at early weaning ( $p < 0.01$ ). As with breast-fed infants though, concentrations of n-butyrate increased significantly through the weaning process and all development stages differed with each other. Acetate decreased significantly at very late weaning from pre-weaning ( $p < 0.01$ ). Propionate did not change significantly between development stages.

Formula-fed infants increased at late and very late weaning ( $p < 0.05$ ) from pre-weaning. Proportions of other SCFA remained similar throughout all development stages.

Previous work has mainly studied pre-weaning infants. These studies have been carried out in Denmark (Rasmussen *et al.*, 1988), Estonia (Siigur *et al.*, 1993) and



the USA (Lifschitz *et al.*, 1990). Neonatal feeding practices differ in these countries to that found in Scotland. Most of these infants had received breast milk from birth and quite often this was given with addition of glucose supplements. Only later did some mothers then also supplement feeding with formula milk. Therefore there was no true formula-fed group. Siigur *et al.*, 1993, showed the main acid produced by breast-fed and those defined as formula-fed infants was acetate. As expected more propionate and butyrate was seen in the formula-fed infants than in the breast-fed infants. Lactic acid, however was not measured in the study by Siigur *et al.* All infants in the Danish study had received breast milk with supplements of formula milk and glucose supplements but again acetate was shown to be the main SCFA produced. Again however, lactate was not measured in these infants. In the study where lactate was measured by Lifschitz *et al.*, 1990, it was found that formula-fed infants had higher propionate but also higher acetate and total SCFA and no difference in lactate. The number of formula-fed infants in the study was small (n=9) and were fed a variety of casein based formula feeds. Studies have shown faecal bacteria of infants fed whey or casein based formula differ significantly (Balmer *et al.*, 1989).

In both breast-fed and mixed fed infants weaning did appear to change the production and pattern of SCFA. Very few differences were seen in the formula-fed group between any of the development stages.

Previous work in weanling mice (Lee and Gemmell, 1972) showed that ingestion of the first solid food corresponded with the appearance of strictly anaerobic fusiform bacilli in the colon alongside a 10,000 fold decrease in coliform bacilli. This was reflected in increased production of SCFA especially butyric acid. Work in rats (Armstrong *et al.*, 1992) found that what was fed at weaning had a significant effect on SCFA produced in later life.

Bacterial populations in 7 breast-fed and 7 formula-fed infants were examined in the first year of life (Stark and Lee, 1982). During the first week of life it was shown that breast-fed and formula-fed infants had similar bacterial populations.

After this the two feeding groups diversified, breast-fed infants had a flora dominated by bifidobacteria whereas formula-fed infants had other anaerobes in addition to bifidobacteria as well as higher counts of facultative anaerobes. In the breast-fed infants introduction of solid food caused a major disturbance in the micro-ecology of the colon. Counts of enterobacteria and enterococci rose sharply and colonisation by *Bacteroides*, clostridia and anaerobic streptococci occurred. These major changes were not apparent when formula-fed infants began to ingest solid food. At 12 months the breast-fed and formula-fed infants had bacterial populations that began to resemble that of the adult.

However, bacteria that exist in the infant colon and its changing nature at weaning are difficult to quantify because of the complexity of infant colonisation and the variation between individual infants. An alternative approach is to study changes in bacterial products for example SCFA which can act as a marker of bacterial activity. Very few studies have investigated SCFA through the weaning process in infants. Midtvedt and Midtvedt, 1992 followed infants from birth to two years of age. This study confirmed that breast-fed infants had mainly acetate prior to weaning with increasing amounts of propionate and butyrate as weaning occurred. The formula-fed infants had higher acetate, propionate and butyrate at pre-weaning and their levels did not change much through the weaning period. Lactate was not measured in this study and the formula-fed group had changed from breast-feeding to formula feeding so are more comparable to our mixed fed group.

Previous authors have stated that less change in bacterial populations were seen during weaning in formula-fed infants (Stark and Lee, 1982). One difference that was seen in the formula-fed infants was the increase in n-butyrate between pre-weaning and all other development stages. Even when the butyrate value was expressed as a percentage there were still significant differences between pre-weaning and both early and late weaning. Butyrate has been shown to be very important in adults but in previous studies butyrate production in infants appears to be very low. This finding holds true in this study and even in formula-fed

infants the amounts produced prior to weaning are much lower than those produced after weaning.

Although differences between breast-fed and formula-fed infants in terms of their faecal flora (Balmer and Wharton, 1989) and SCFA (Edwards *et al.*, 1994) is well established very little is known about infants that receive a mixture of feeding. Mixed feeding can include transfer to formula milk after a period of breast-feeding or a combination of breast milk and formula milk. It is not known whether this group has patterns like that of the breast-feeding or formula-feeding group. There has been a study to suggest that if one bottle of formula milk is given this causes the flora to change from being that of a breast-fed infant to that of a formula-fed infant (Bullen, 1977).

Mixed feeding is prevalent in Scotland and the UK as a whole. In Scotland, only half of all mothers breast-feed at birth (OPCS, 1990). Since 1974 there has been an official recommendation that mothers breast-feed their infants. It has been suggested that mothers breast-feed preferably for four to six months but at least for the first few weeks of life. It has been shown that breast-feeding for the first 13 weeks protects infants against gastrointestinal illness and respiratory infection in the first year of life (Howie *et al.*, 1990). The 1990 OPCS figures show that many mothers do not achieve the target of four to six months or even 13 weeks. As many mothers, because of breast-feeding initiatives, do start breast-feeding and then for a variety of reasons stop a large number of infants can be considered mixed fed. By 4 months 30% of infants in Scotland will have received a mixture of breast and formula milk (White *et al.*, 1992). Any infant that has mixed feeding before 13 weeks may not achieve the full protective effect of breast-feeding that was outlined by Howie *et al.*, 1990.

Comparisons between the three feeding groups showed that mixed fed infants were a distinct group and had characteristics that were different from breast-fed or formula-fed infants. Mixed fed infants had significantly more propionate and butyrate ( $p < 0.001$ , 0.05) at pre-weaning than breast-fed and significantly less

lactate ( $p < 0.001$ ). The mixed fed group had less butyrate than the formula fed group at pre-weaning ( $p < 0.05$ ) and more lactate than them at early weaning, although this was not significant. Formula-fed infants had significantly more propionate and butyrate and less lactate at pre- ( $p < 0.001$ ) and early weaning ( $p < 0.01$ ) than breast-fed infants. However by late and very late weaning differences between the feeding groups had disappeared.

## 5.6 CONCLUSIONS

The onset of weaning appeared to change the production and pattern of SCFA in both breast-fed and mixed fed infants, however this was not the case in formula-fed infants. Total SCFA were significantly lower at pre-weaning than at early, late or very late weaning for breast-fed and mixed fed infants whereas in formula-fed infants there was no significant difference in the amount of total SCFA and lactate produced through any of the development stages. This would correspond to previous work that shows that formula-fed infants even prior to weaning have a faecal flora more like that of an adult and their microflora undergoes less change at weaning and older ages than that of breast-fed or mixed fed infants.

The mixed fed infants were a distinct group from the breast-fed and formula-fed infants in terms of their faecal SCFA and had characteristics from both groups. This suggests that the physiological development of the mixed fed infants differ from exclusively breast-fed and exclusively formula-fed infants. These differences are present up until late weaning when the groups converge in terms of their faecal SCFA profiles. As the late weaning samples were from infants aged 9 months to a year old this may suggest that the production and profile of SCFA become similar in all infants and more like that of an adult at an earlier age than previous literature suggests.

## **Chapter 6**

### **Faecal Starch and Faecal Fat Excretion**

This chapter describes the faecal fat excretion in breast-fed and formula-fed infants and the faecal starch excretion of infants in all the feeding groups.

## 6.1 INTRODUCTION

Birth and weaning are critical periods in the infants' development because of the changes in diet that infants undergo at these times. At birth the infant switches from processing dilute amniotic fluid to processing milk and at weaning the diet of the infant undergoes a major change as new foods are introduced (Buddington, 1993), including complex carbohydrates such as starch. Little is known about the digestibility and fermentability of foods in infancy.

The newborn infant does not have the ability to digest complex carbohydrates because of immature intestinal function. Several of the factors necessary for the digestion of fat and carbohydrate (pancreatic enzymes, bile acids) are not present at adult levels in the infant at birth (Lebanthal *et al.*, 1983). The development of intestinal function continues for several months after birth (Guandalini, 1991).

Prior to weaning the major energy sources of the infant are lactose and fat, when weaning begins the amount of energy supplied from fat drops from 50%-30% (Giovanni and Agostoni, 1991) and starch becomes the primary source of energy. Malto-dextrins may be present in formulas and weaning foods. Starch used in first weaning foods varies in its digestibility, for instance rice starch is rapidly digestible. Commercial weaning foods, however, are subject to sterilising techniques (similar to autoclaving) that may considerably increase the amount of resistant starch because of retrogradation. Cooked potato may be used as a home-prepared weaning food and is rapidly digestible but if left to cool after cooking (which is often the case before feeding an infant) it may become retrograded and resistant to digestion.

Interest in starch and fat digestibility arises from two main considerations: to what extent can starch and fat be considered caloric sources and how well are they

digested in the infant. If starch and fat are not digested they pass through to the colon where they are available for bacterial metabolism or they are excreted in the faeces. If excreted in the faeces this may represent a loss of energy to the infant.

### **6.1.1 Starch**

Carbohydrate can be classified into monosaccharides, disaccharides and complex carbohydrates. Complex carbohydrates consist of the oligosaccharides (3-10 sugar residues) and polysaccharides (more than 10 sugar residues). Starch is the major polysaccharide of the human diet and is the main storage polysaccharide of dietary staples. Starch consists of two main polysaccharides derived from glucose. Amylose is a linear chain of glucose units with  $\alpha$ 1-4 linkages and amylopectin is a branched chain polymer with 15-30  $\alpha$ 1-4 linked glucose units joined by  $\alpha$ 1-6 linkages. Although amylopectin dominates in most starches, the relative amounts of amylose and amylopectin vary among different plant sources.

In starch granules, the amylose and amylopectin chains are arranged in a semicrystalline structure, this makes them insoluble in water and inhibits their digestion by pancreatic amylase. X-ray diffraction studies (Katz, 1934) have shown there are three crystalline forms, A (cereal starches), B (tuber starches) and C (pea and bean starches, considered to be a mixture of A and B), which differ in their digestibility. When starch granules are heated in the presence of moisture the crystalline structure is broken down and the polysaccharide chains take up a random conformation. This causes swelling of the starch granule and thickening of the surrounding matrix (known as gelatinisation) which allows the starch to become available to digestive enzymes. On cooling the gelatinised starch recrystallises, known as retrogradation which renders them resistant to enzymic digestion. Balance studies have shown that normal, healthy 1-month old infants are able to absorb 10g/ day of cooked wheat, corn, tapioca or potato starch. They have a greater ability to digest cooked rice starch, quantities of up to 40g/ day can be absorbed. At 30 months of age, 20-25g of the same starches can be absorbed. By the age of 1-2 years 99% of 170g/ m<sup>2</sup>/ day of cooked wheat or potato starch

are absorbed although only 93% of non cooked starch can be absorbed (Auricchio *et al.*, 1968; De Vizia *et al.*, 1975).

Starches have also been classified on the basis of their digestibility *in vitro* (Table 6.1). This gives an indication of the behaviour of starchy foods in the intestinal tract. The amounts of RDS, SDS and RS (Englyst *et al.*, 1992) found in foods vary and depend not only on the source of starch but the type and extent of processing they have undergone. Resistant starch is defined as ‘the sum of starch and products of starch degradation not absorbed in the small intestine of healthy subjects’ (Asp, 1992). The type of starch available in formulas and infant weaning foods can differ greatly, especially in commercial weaning foods where the processing and sterilising techniques may alter the starch drastically.

The effect these starches have on the infant *in vivo* is largely unknown but may have effects on energy absorption and growth potential.

Starch may be difficult to digest in infancy due to the enzymes required are either not present or only present at low levels. Pancreatic amylases are low or absent up to 4 months of age and only reach significant levels of activity at about 1 year of age (Zoppi *et al.*, 1972). At this age therefore, salivary amylase and breast milk amylase are more important. Salivary amylase, present at birth, rises to adult concentrations between 6 months and one year (South, 1971). A large amount of salivary amylase may be inactivated by hydrochloric acid in the stomach but infants do hydrolyse some starch.

Salivary amylase remains active in the stomach of the new-born allowing starch digestion to occur (Hodge *et al.*, 1983). Most digestion in the first weeks of life is due to the presence of glycosidase and  $\alpha$ -glucoamylase present in the brush border of the small intestinal enterocytes (Guandalini, 1991). These enzymes are active within the first few days of life (Lebenthal and Lee, 1980). They are able to split the glucose from the terminal end of amylose and amylopectin, especially those



containing 5-9 residues of glucose. Longer polymers such as those found in starch are more difficult to digest in the first two months of life.

Any carbohydrate that is not digested and absorbed in the small intestine will pass to the colon. It then becomes available for the bacteria to ferment, this produces SCFA which are an available source energy. It is now considered that the fermentation of carbohydrate in adults has an energy value of 2 Kcal/g (Livesey, 1990). However, if the carbohydrate escapes both digestion in the small intestine and fermentation in the colon it will be excreted in the faeces and not contribute to energy.

### **6.1.2 Fat**

Fat is a major source of energy for the infant contributing 40-50% of the calories in human milk and most infant formulas. New-born breast-fed infants consume 5-7.5 g fat/ kg body weight/ day, which is the equivalent of 350-525g/ day for an adult (Harries, 1982). From fetus to new-born there is a transition from carbohydrate to fat as the major energy source. At birth the pancreas and enteropathic circulation of bile salts are still immature but even a premature infant can absorb 65-80% of ingested fat.

The more efficient digestion of fat by breast fed infants is thought to be the result of the presence bile salt stimulated lipase in human milk (Hernell, 1975) and also the chemical structure of breast milk triacylglycerol is more favourable to fat hydrolysis. There is now evidence that long chain fatty acids may play an important role in fat hydrolysis and absorption (Bernback *et al.*, 1989; Borgstrom, 1980). The exclusion of long chain polyunsaturated fatty acids (LCPUFA) from formula fed infants may negatively influence fat hydrolysis, increase calcium soap formation and increase stool hardness. The prevalence of hard stools was significantly reduced in infants fed a formula supplemented with LCPUFA.

Milk triacylglycerol is the principal energy source, the saturated fats, palmitic and stearic acid are also sources of energy. The long chain polyunsaturated fatty acids, arachadonic acid and docosahexaenoic acid, are essential components of membranes and are thought to be important in neurological development (Farquharson *et al.*, 1992).

In older infants and adults, dietary fats are hydrolysed by pancreatic lipases in the small intestine. Lipolysis products are then solubilised by the action of the bile salts. In the newborn infant pancreatic and hepatic functions are not fully developed and pancreatic lipase and bile salts are only found at low concentration (Zoppi *et al.*, 1972). Synthesis of bile does occur but the bile acid pool is half the size of that of an adult (Watkins, 1974). Lingual and gastric lipase (Hamosh and Burns, 1977) and milk lipases (Hall and Muller, 1982) contribute to the infants' ability to digest fats. In breast-milk bile-salt stimulated lipase plays a role in fat digestion and absorption. The digestion and absorption of fat in formula-fed infants may be less efficient.

Despite a large proportion of dietary fat being absorbed a substantial amount is not digested and reaches the colon. There has been little investigation on the fate of the fat in the colon. However recent studies have suggested that it may be important on the microbiology (Thompson and Spiller, 1996) and function (Spiller *et al.*, 1986) of the colon and may play a part in the aetiology of colonic cancer. Colonic bacteria can metabolise dietary fatty acids which are potentially toxic and can stimulate colonocyte proliferation (Bull *et al.*, 1988).

**Table 6.1 Classification of starch**

Type of starch	Description	Occurrence	Digestibility
Rapidly digestible starch (RDS)	Mainly amorphous and dispersed starch	Freshly cooked starchy foods	Rapid
Slowly digestible starch (SDS)	Starch poorly accessible to enzymes	Most raw cereals	Slow but complete
Resistant starch (RS)	Starch that avoids digestion in the small intestine and becomes available for fermentation in the large intestine		
I	Physically inaccessible starch	Partly milled grains and seeds	Resistant
II	Resistant starch granules	Raw potato and banana	Resistant
III	Retrograded amylase	Cooked potato, bread, cornflakes	Resistant

## **6.2 METHODS**

The main aim of the work described in this Chapter was to investigate the effect of feeding practice on faecal excretion of fat and starch of infants in the first year of life.

### **6.2.1 Faecal Fat**

Faecal fat was measured in subsets of infants from the main sample group. Excreted fat was determined in breast-fed (n=10) and formula-fed infants (n=10). Measurements of faecal fat were made at pre weaning, when exclusively breast or formula milk fed (1-2 months), four weeks after first giving solid food (early weaning) and late weaning (9 months –1 year). Faecal fat excretion was measured at each development stage to compare both between development stages and between infant feeding groups.

### **6.2.2 Faecal Starch**

Faecal starch was measured in subsets of infants from the main sample group. Measurements were made in 10 breast-fed and 10 formula-fed infants at pre-weaning (exclusively breast or formula milk), four weeks after first giving solids (early weaning), late weaning (9 months - 1 year) and very late weaning (18 months – 2 years). In addition faecal starch was measured in eleven mixed fed infants from the main sample group. These were measured at early weaning, late weaning and very late weaning. Faecal starch excretion was measured at each time point to see if excretion of starch differed, either between the time points or between the feeding groups.

Methods for collection and determination of faecal fat and faecal starch are described in detail in Chapter 2.

### **6.2.3 Definitions of developmental stages and feeding method**

Infants were classified as breast-fed if they had been exclusively breast-fed since birth until the start of giving solids. At pre-weaning, other than breast milk, they may have been given water. Formula-fed infants had been exclusively formula-fed from birth. The mixed fed infants were a varied group who had been breast-fed and then been transferred to formula milk or if they received a mixture of breast and formula milk in any ratio. Details of mixed fed infants are given in Chapter 4, Table 4.2.

The three developmental stages were chosen to show the progression of the diet from birth to early childhood. Pre-weaning represented an exclusively milk diet, breast or formula. Early weaning was selected as four weeks after weaning. This was to give a uniform point not dependent on age, weaning had been started and had been ongoing for the same length of time in each infant. This was to resolve problems of selecting an age for early weaning where some mothers may have been weaning for one week whereas others may have been giving solids for several weeks. Although the rate at which mothers progress with giving solid foods may differ from infant to infant it was thought that 4 weeks after first giving solid food may be more comparable for all infants. At this stage of early weaning the infants were receiving liquidised and pureed foods. The final development age was late weaning where more structured chopped and whole foods would be given as the teeth and locomotor functions of the infant would be developing.

Food intake for the three days prior to infants giving stool samples at early and late weaning are described in Chapter 4, Table 4.12 (subject numbers: breast-fed 3, 6, 9, 12, 13, 14, 15, 17, 18, 28; formula-fed 1, 2, 3, 4, 5, 6, 7, 9, 12, 14; mixed fed 1, 2, 4, 6, 8, 9, 10, 11, 13, 16, 17).

## 6.2.4 Statistics

Comparisons of longitudinal data were by the one-sample Wilcoxon test. To compare between feeding groups statistical analysis was by Kruskal-Wallis followed by Mann-Whitney.

## 6.3 RESULTS

### 6.3.1 Longitudinal comparison of faecal starch excretion

In breast-fed infants the amount of faecal starch excreted (Table 6.2) at pre-weaning was significantly lower than for all other development stages ( $p < 0.05$  compared with early and late weaning,  $p < 0.01$  compared with very late weaning). Although at early weaning faecal starch was still low this was not significantly lower than late or very late weaning. In formula-fed infants similarly at pre-weaning faecal starch excretion was lower than any other development age ( $p < 0.05$  compared with late weaning,  $p < 0.01$  compared with early and very late weaning). Pre-weaning data for the mixed fed infants were not analysed. The amount of faecal starch excreted at very late weaning by mixed fed infants is significantly lower compared with late weaning ( $p < 0.05$ ). Figures 6.1, 6.2 and 6.3 show the faecal starch at each weaning stage for individual infants.

**Table 6.2 Faecal starch excreted by breast-fed, formula-fed and mixed fed infants**

	Pre-weaning		Early weaning		Late weaning		Very late weaning	
	Median	Range	Median	Range	Median	Range	Median	Range
BF	0.09	0 – 0.25	0.6*	0.02 – 1.49	1.38*	0.13 – 2.75	1.04**	0.12 – 5.37
FF	0.02	0 – 0.22	0.2**	0.02 – 2.33	1.12*	0.32 – 2.92	0.57**	0.14 – 2.32
MF	ND	ND	0.74	0.26 – 3.43	1.17	0.55 – 3.28	0.5*	0.0 – 2.1

$p < 0.05$ , \*\*  $p < 0.01$  compared with pre-weaning, \*  $p < 0.05$  compared with late weaning

BF=Breast-fed, FF=Formula-fed, MF=Mixed fed

Figure 6.1 and 6.2 Faecal starch excretion in breast-fed (top diagram) and formula-fed infants (bottom diagram).

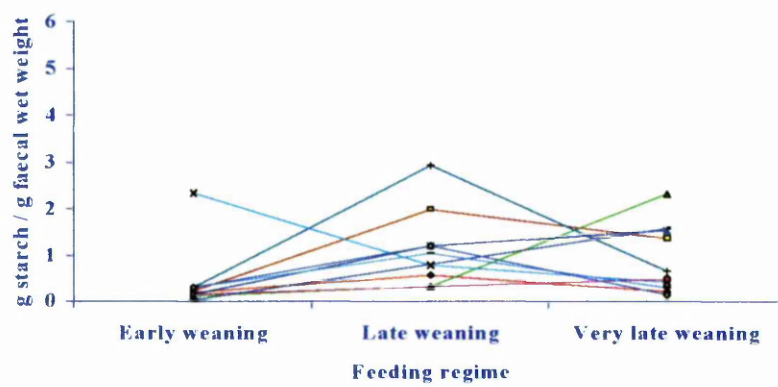
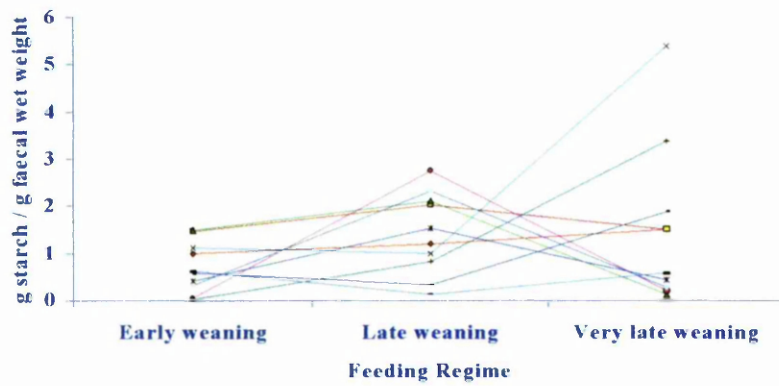
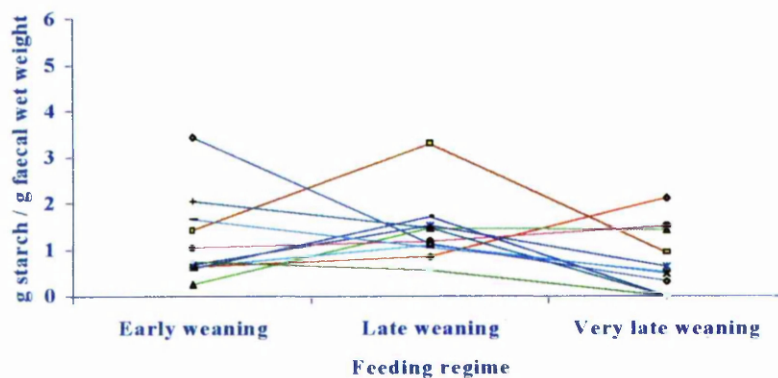


Figure 6.3 Faecal starch excretion in mixed fed infants



#### 6.3.1.1 Comparisons of faecal starch excretion between breast-fed, formula-fed and mixed fed infants

The only significant difference between the three feeding groups was seen at early weaning, where mixed fed infants excreted significantly more faecal starch than formula-fed infants (Table 6.3). Although breast-fed infants also excreted more faecal starch than formula-fed infants at this development stage, the difference did not reach statistical significance. At late weaning the values for the three feeding groups were similar. No faecal starch analysis was made on samples from mixed fed infants before weaning. Breast-fed infants had higher faecal starch excretion than formula-fed infants at pre-weaning but this was not significant. At very late weaning, whilst formula-fed and mixed fed had similar values, breast-fed infants excreted more faecal starch but again this did not reach significance.



**Table 6.3** Faecal starch excretion in breast-fed, formula-fed and mixed fed infants

	Pre-weaning		Early weaning		Late weaning		Very late weaning	
	Median	Range	Median	Range	Median	Range	Median	Range
BF	0.09	0 – 0.25	0.6	0.02 - 1.49	1.38	0.13 - 2.75	1.04	0.12 - 5.37
FF	0.02	0 - 0.22	0.2	0.02 – 2.33	1.12	0.32 – 2.92	0.57	0.14 – 2.32
MF	ND	ND	0.74**	0.26 – 3.43	1.17	0.55 – 3.28	0.5	0.0– 2.1

♦♦ p<0.01 compared with formula-fed infants.

BF=Breast-fed, FF=Formula-fed, MF=Mixed fed

### 6.3.2 Longitudinal comparison of faecal fat excretion

Faecal fat excretion was low for breast-fed infants throughout the development stages (Table 6.4) and there were no differences between any of the stages. In formula-fed infants excretion of faecal fat was similar in pre and early weaning and then decreased in late weaning. The amount excreted at late weaning was significantly lower than at pre-weaning ( $p<0.01$ ).

**Table 6.4** Comparison of excretion of faecal fat through the development stages in breast-fed and formula-fed infants

	Pre-weaning		Early weaning		Late weaning	
	Median	Range	Median	Range	Median	Range
BF	0.08	0.05 – 0.39	0.06	0.04 – 0.25	0.1	0.03 – 0.34
FF	0.41	0.23 – 0.91	0.30	0.09 – 0.52	0.12**	0.03 – 0.29

\*\* p<0.01 compared with pre-weaning. BF=Breast-fed, FF=Formula-fed

#### 6.3.2.1 Comparisons of faecal fat excretion between breast-fed and formula-fed infants

When the two feeding groups were compared, breast-fed infants had significantly lower faecal fat excretion than formula-fed infants ( $p<0.001$ ) at pre and early weaning. By late weaning both groups of infants had similar faecal fat excretion (Table 6.5).

**Table 6.5 Comparison of breast-fed and formula-fed infants for faecal fat excretion**

	Pre-weaning		Early weaning		Late weaning	
	Median	Range	Median	Range	Median	Range
BF	0.08	0.05 – 0.39	0.06	0.04 – 0.25	0.1	0.03 – 0.34
FF	0.41***	0.23 – 0.91	0.30***	0.09 – 0.52	0.12	0.03 – 0.29

\*\*\* p<0.001 compared with breast-fed infants. BF=Breast-fed, FF=Formula-fed

## 6.4 DISCUSSION

Faeces contain food material that has escaped both digestion and fermentation in the colon. If starch or fat are undigested and unabsorbed in the small intestine they will pass to the colon. If they remain unmetabolised by the colonic bacteria they are excreted in the faeces.

Carbohydrates that pass through to the colon are fermented by the colonic bacterial flora producing SCFA and gases. SCFA are readily absorbed and can be utilised as a source of energy by the colonocytes or the host. This process is known as ‘colonic scavenging’ (Kien *et al.*, 1990; Lifschitz, 1996) and is an important mechanism for salvage of lactose in infants and of complex carbohydrates in older infants and children. The SCFA also promote water absorption thereby preventing diarrhoea.

There may be two mechanisms whereby starch which enters the colon arrives in the stool. If the amount of carbohydrate arriving in the colon is increased then fermentation may not be complete and so carbohydrate passes into the stool. Alternatively complex carbohydrates may arrive in the colon and bacteria may not be adapted to ferment it thus it will pass through unmetabolised. Therefore young infants who have an incomplete development of the flora or an immature colon will not be able to cope with excessive carbohydrate. The unmetabolised carbohydrate will pull water with it and lead to increased stool output and possible diarrhoea.

Before weaning breast-fed infants ingest human milk therefore starch should not be seen in the faeces, however, in formula-fed infants where starch may be present in the formula-milk starch may not be digested or fermented because of the immature gastrointestinal tract and the undeveloped colonic flora.

As weaning begins the infants of all feeding groups are exposed for the first time to a range of new foods, including complex carbohydrates. A significant amount of this will escape digestion because of immature pancreatic exocrine function (McClellan and Weaver, 1993) and the lack of chewing ability. The colonic salvage of energy from this material entering the colon may contribute significantly to the energy needs of the infants. In adults energy gained from fermentation of carbohydrate is estimated as 8.4 kJ (2 kcal)/ g, unabsorbable carbohydrate (Livesey, 1990). If colonic fermentation capacity is poorly developed then the unfermented starch is likely to increase stool output and increase faecal energy losses.

Starch has been shown to be present in faeces of children up to three years of age (Verity and Edwards, 1994). This coupled to findings of the slow rate of development of other bacterial activities such as degradation of mucin, conversion of bilirubin to urobilinogen, metabolism of cholesterol to coprostanol and inactivation of faecal tryptic activity (Midtvedt *et al.*, 1988) suggests that development of fermentation capacity is slow.

In this study faecal starch was higher at all stages of weaning than at pre-weaning for breast-fed and formula-fed infants. Mixed fed infants were not analysed for faecal starch for pre-weaning. The amount of starch being excreted in the faeces increased from pre-weaning to late weaning (for all feeding groups) and after peaking at late weaning dropped slightly but not statistically significantly in very late weaning. This increase in starch through the weaning age parallels the increasing amount of starch that the infant is obtaining in the diet. Levels excreted in the faeces may suggest that the colonic bacteria have not adapted enough to be able to ferment all the starch

coming into the colon at these ages. At very late weaning starch excretion begins to drop and this may be because colonic bacteria are more adapted to be able to ferment the starch that comes into the colon. Alternatively less starch is actually entering the colon because as the gut is maturing more starch is being digested.

When the feeding groups are compared it can be seen that similar amounts of starch were produced by all three feeding groups at late and very late weaning. At early weaning there was a significant difference between mixed fed and formula-fed infants, mixed fed infants excreted higher amounts of starch, this was also the case when comparing breast-fed and formula-fed infants but it did not reach statistical significance. This may be an indication that the bacterial flora of breast-fed and mixed fed infants develops more slowly than formula-fed infants but that by late weaning they 'catch-up'.

The amount of fat excreted by breast-fed infants remains very low throughout the weaning stages and there were no significant differences between any development stage. Formula-fed infants, in contrast, excrete significantly more fat at pre-weaning than at late weaning. This suggests that by late weaning the mechanism for digesting and absorbing fat from the small intestine has matured. Breast-fed infants excreted significantly less fat than formula-fed infants in pre- and early weaning ( $p < 0.001$ ). This correlates with the better mechanisms for fat digestion and absorption in breast-fed infants.

There has been evidence that colonic bacteria can metabolise fats (Mackie *et al.*, 1991). Colonic bacteria are able to metabolise dietary fats to hydroxy fatty acids (Kim and Spritz, 1968). Hydroxy fatty acids are potentially toxic and stimulate colonocyte proliferation (Bull *et al.*, 1988).

The differences in faecal fat excretion both longitudinally and between breast-fed and formula-fed was probably due to the increased ability of breast-fed infants to digest

fat because of the type of fat in breast milk. The high digestibility of human milk has been shown to be related to the presence of bile salt-stimulated lipase in human milk (Hernell and Blackberg, 1982), the structure of the milk fat globule (Gaull *et al.*, 1982) and to the sn-2 configuration of palmitic acid in the triglyceride (Filer *et al.*, 1969). The formula milk fats are composed of vegetable fat which have sn-1,3 position in the triglyceride. The bile salt-stimulated lipase and the structure of the milk fat globule improve triglyceride lipolysis in breast-fed infants. The sn-2 configuration of palmitic acid allows the absorption of palmitate as the 2-monoglyceride from human milk rather than the free acid.

Differences in lipids between formula- and breast-fed infant stools has been shown to be almost entirely due to fatty acids, mainly C16:0 and C18:0 excreted as soaps (Quinlan *et al.*, 1995). The authors suggested that this was due to the infants handling of saturated fatty acids. Calcium fatty acid soaps were shown to be related to stool hardness and may, in part, explain the greater stool hardness in formula-fed infants compared with breast-fed infants.

Starch and fat lost in the faeces may represent a significant amount of energy loss for the infant. Although there is very little data on the stool output in early life and our data only represents a 'snapshot', it may be useful to roughly quantify these faecal energy losses. 1 g of starch and 1g fat releases 4 kcal and 9 kcal of energy respectively. From this it is possible to calculate the amount energy lost through starch and fat excretion. According to Weaver *et al.*, (1988b) infants, by 16 weeks of age, pass an average of two stools per day of mean size 10mls. If each ml of stool is converted to grammes this is equivalent of 20g of stools passed by infants each day. As the infant matures this stool will increase, so by 1 and 2 years (late and very late weaning respectively) they may be passing larger volumes of stool. As there is no available data for older children for the sake of this thesis, I will assume quantities of 100g per day. Estimated average energy requirements for energy (DOH, 1999) for males 4-6 months is 690 kcal and for girls 645 kcal. An average of these two

figures, 668 kcal, is taken for energy intake for early weaning. Similar averages for energy intakes are taken for late weaning, 893 kcal, and very late weaning, 1198 kcal. Estimated faecal energy lost is shown in Tables 6.6, 6.7, 6.8.

**Table 6.6 Percentage of daily energy lost through faecal starch excretion**

	Early weaning			Late weaning			Very late weaning		
	Median	Min	Max	Median	Min	Max	Median	Min	Max
<b>Breast-fed</b>									
Faecal starch g/100g	0.6	0.02	1.49	1.38	0.13	2.75	1.04	0.12	5.37
Energy lost kcal	0.48	0.02	1.19	5.52	0.52	11.00	4.16	0.48	21.48
% energy lost	0.07	0.00	0.18	0.62	0.06	1.23	0.35	0.04	1.79
<b>Formula-fed</b>									
Faecal starch g/100g	0.2	0.02	2.33	1.12	0.32	2.92	0.57	0.14	2.32
Energy lost kcal	0.16	0.02	1.86	4.48	1.28	11.68	2.28	0.56	9.28
% energy lost	0.02	0.00	0.28	0.50	0.11	1.31	0.19	0.05	0.77
<b>Mixed fed</b>									
Faecal starch g/100g	0.74	0.26	3.43	1.17	0.55	3.28	0.50	0.00	2.10
Energy lost kcal	0.59	0.21	2.74	4.68	2.20	13.12	2.00	0.00	8.40
% energy lost	0.09	0.03	0.41	0.52	0.25	0.11	0.17	0.00	0.70

Values are given for faecal starch excretion g/ 100g faecal wet weight. This is converted to energy lost by 1g starch is equivalent to 4 kcal of energy and then divided by 5 to give an energy lost per 20g daily stool output for early weaning or left as per 100g for late and very late weaning. This is then converted to percentage of energy lost of estimated average requirements.

**Table 6.7 Percentage of daily energy lost through faecal fat excretion**

	Early weaning			Late weaning		
	Median	Min	Max	Median	Min	Max
<b>Breast-fed</b>						
Faecal fat g/100g	0.06	0.04	0.25	0.1	0.03	0.34
Energy lost	0.11	0.07	0.45	0.9	0.27	3.06
% energy lost	0.02	0.01	0.07	0.10	0.03	0.34
<b>Formula-fed</b>						
Faecal fat g/100g	0.30	0.09	0.52	0.12	0.03	0.29
Energy lost	0.54	0.16	0.94	1.08	0.27	2.61
% energy lost	0.08	0.02	0.14	0.12	0.03	0.29

Values are given for faecal fat excretion g/ 100g faecal wet weight. This is converted to energy lost by 1g starch is equivalent to 9 kcal of energy and then divided by 5 to give an energy lost per 20g daily stool output for early weaning or left as per 100g for late weaning. This is then converted to percentage of energy lost of estimated average requirements.

**Table 6.8 Estimation of percentage energy lost daily through faeces**

	Early weaning			Late weaning		
	Median	Min	Max	Median	Min	Max
Breast-fed	0.09	0.01	0.25	0.72	0.09	1.57
Formula-fed	0.10	0.02	0.42	1.22	0.20	1.60

Figures are an addition of faecal starch and fat excretion.

The maximum amount of energy lost for any infant would be 1.79 % of daily energy needs (calculated from the maximum starch excreted by one infant in the breast-fed group at early weaning). Although these are both estimates and a 'snapshot' of faecal losses it can be seen that the approximate energy lost through faecal losses from fat and starch excretion is minimal and would therefore not represent a problem to the health of the infants.

It was difficult from the limited dietary data collected during this study to relate food intake with starch and fat excretion. In the case of individual infants, it was seen that two infants who were excreting high quantities of starch had received high starch food for the three days prior to giving the faecal sample. An infant in the mixed fed group who had excreted 3.43 g starch / 100 g faecal wet weight at early weaning had received a high proportion of vegetables for the three days prior to giving the sample. Similarly an infant in the formula-fed group who had a value of 2.33g starch excreted / 100g faecal wet weight had consumed mashed banana on the three days prior to giving the sample which may, depending on the ripeness of the banana, contain large amounts of resistant starch (Englyst *et al.*, 1992). In contrast there were no obvious relationship between dietary fat and faecal fat.

## 6.5 CONCLUSIONS

Faecal starch was excreted from early weaning onwards in all groups of infants. The amount of starch excreted increased from pre-weaning. There was a slight, but not statistical decrease at very late weaning. This increased faecal starch correlated with an increase in dietary starch. This suggests that both gut function and colonic

bacteria have not reached levels of maturity to be able to digest and ferment available starch. Starch appearing in the faeces may represent a significant loss of energy.

There was increased faecal starch at early weaning in mixed fed and breast-fed infants than formula-fed infants, this only reached significance with mixed fed infants. By late and very late weaning differences between infant feeding groups had disappeared. This suggested that mixed fed infants in particular, but also breast-fed infants to a lesser extent, have a decreased ability to digest and ferment starch at early weaning. This may indicate that the bacterial flora in these two groups of infants develops more slowly than in formula-fed infants. However, rough estimations of percentage energy lost daily through faecal starch was very little.

Faecal fat excretion was significantly less in pre- and early weaning in breast-fed and formula-fed infants. This correlates with the better mechanism of breast-fed infants for fat digestion and absorption. Although the percentage energy lost through daily fat excretion has been shown by rough estimations to be negligible, the adverse effects of fat passing through to the colon and faeces may be of more importance.

*I would like to acknowledge the help of Sheila Khanna for analysis of faecal starch of breast-fed infants at late weaning,, and Keira Farley for analysis of faecal starch in mixed fed infants.*



## **Chapter 7**

### **Development of Fermentation Capacity in Breast-Fed, Formula- Fed and Mixed Fed Infants, a Longitudinal Study**

## 7.1 INTRODUCTION

In this chapter the longitudinal study of development of fermentation capacity will be discussed and compared with the results of the cross-sectional study (Chapter 2). Carbohydrates that escape digestion and absorption in the small intestine pass to the colon where they are anaerobically metabolised by the colonic bacteria to produce short chain fatty acids (SCFA) mainly acetate, propionate and n-butyrate (Edwards *et al.*, 1994). Smaller amounts of the branched chain fatty acids are produced by bacterial anaerobic metabolism of amino acids (Macfarlane *et al.*, 1992). SCFA have many potential roles (see Chapter 1), including acting as a source of energy, which may provide a form of 'colonic salvage' and prevent osmotic water loss (Kien *et al.*, 1990).

Carbohydrate fermentation in adults has been well investigated but knowledge of the colonic flora in infants and how they respond to new substrates at weaning is limited. Early events in the bacterial colonisation of the gut, at birth and at weaning, may be critical in determining the health of the infant as well as establishing the colonic flora of the adult.

Previous studies have shown that diet in early life influences the colonisation of the gut. Prior to weaning, breast-fed infants have a colonic microflora dominated by bifidobacteria and lactobacilli, whereas formula-fed infants have more enterobacteriaceae and anaerobic bacteria, resembling the flora of adults more closely (Balmer and Wharton, 1989). This difference in colonic flora is reflected in the pattern of SCFA produced, breast-fed infants produce predominantly acetate and lactate whereas formula-fed infants produce acetate and more propionate and butyrate (Edwards *et al.*, 1994). There is a lack of information about the colonic flora of mixed fed infants but the SCFA profile appears to be dominated by acetate and propionate (Chapter 5) which is in between that of breast-fed and formula-fed infants.

Although butyrate is considered very important in adults (Chapter 1) very little is produced in infants. This may mean it is not critical for the infant colon or that most butyrate produced is absorbed before it reaches the faeces.

The simplicity of the infant flora limits its fermentation capacity for complex carbohydrates. As the infant matures, its colonic flora becomes more complex. Weaning may be critical for the development of the flora. The relatively simple flora matures and diversifies through the introduction of new substrates at weaning until an adult flora is established. The bacterial flora of the adult until recently was thought to consist of over 400 species dominated by bacteroides, eubacteria, and bifidobacteria resulting in  $10^{11}$  (Moore *et al.*, 1978). Recent research using molecular techniques, using probes for rRNA and bacterial DNA, have suggested that a major proportion of the colonic microflora is still unidentified (Vaughan *et al.*, 2000).

Before weaning, breast-fed infants ingest only human milk but a significant amount of carbohydrate, lactose and fructo-oligosaccharides, may still escape digestion and absorption because of the immature activity of pancreatic and brush border enzymes (McClean and Weaver, 1993). As weaning begins, infants are exposed for the first time to a number of complex carbohydrates. A significant amount of starch will escape digestion because of the lack of chewing ability and immature pancreatic exocrine functions in these infants (McClean and Weaver, 1993). This starch and any ingested dietary fibre will enter the colon where it is available for fermentation by the bacteria. If colonic fermentation capacity develops rapidly, the colonic salvage of energy may contribute significantly to daily energy needs, up to 2 kcal g unabsorbable carbohydrate (Livesey, 1990) in these infants. If colonic fermentation capacity is restricted the unabsorbed and unfermented carbohydrate is likely to increase stool output and faecal energy losses.

Fermentation is dependent on both the composition of the flora and the carbohydrate available. These two factors determine the patterns of SCFA production. Bacteria

produce characteristic SCFA, which can be used in their identification. Not all SCFA are produced by each species (Macy and Probst, 1979; Madelstam *et al.*, 1982; Table 7.1).

**Table 7.1      Products of fermentation by bacterial genera**

<b>Bacteria Genera</b>	<b>SCFA</b>
Bacteroides	acetate, propionate, succinate
Eubacterium	acetate, butyrate, lactate
Bifidobacterium	acetate, lactate
Lactobacillus	acetate, lactate
Clostridium	acetate, propionate, butyrate, lactate
Streptococci	acetate, lactate
Ruminococcus	acetate, lactate, succinate

The pattern of SCFA produced during fermentation in mixed culture is dependent mainly on the carbohydrate substrate (Edwards and Rowland, 1992). Acetate is usually the predominant SCFA produced with all fermentable polysaccharides. The percentage acetate produced can vary from 50% with arabinogalactan and starch to over 80% with xylan and pectin (Englyst *et al.*, 1987). Some carbohydrates produce proportionally more butyric acid (29% with starch, Englyst *et al.*, 1987; 38% with resistant starch, Englyst and Macfarlane, 1986; 22.5% with oat bran, McBurney and Thompson, 1987). In contrast, proportionally more propionate is produced with guar gum (27.2%, Adiotomre *et al.*, 1990), ispaghula (26.3%, Edwards, unpublished data) and arabinogalactan (42%, Englyst *et al.*, 1987). In addition, each bacterial species can ferment only particular substrates. Most bacteria can ferment the mono- and disaccharides but some complex carbohydrates are only fermented by certain genera (Edwards and Rowland, 1992). *Bacteroides fragilis* can ferment starch but not pectin, however *Bacteroides ovatus* will ferment hemicelluloses, starch and pectin. *Bifidobacterium adolescentis* will ferment hemicelluloses and starch, whilst some strains of *Bifidobacterium longum* and *Bacteroides* can also ferment gum arabic, guar and tragacanth (Salyers *et al.*, 1977; McCarthy and Salyers, 1988; Tomlin *et al.*, 1988).

Studies in animals have shown that the pattern of weaning, both the timing and foods introduced, determine the development of the flora and the profiles of fermentation products. In weanling mice (Lee and Gemmell, 1972) ingestion of the first solid food corresponded with the appearance of strictly anaerobic fusiform bacilli in the colon alongside a 10,000-fold decrease in coliform bacilli. This was reflected in increased production of SCFA especially butyric acid. Studies in rats (Armstrong *et al.*, 1992) have suggested that the exposure of the microflora to a particular substrate at weaning may determine the response to that substrate in adult life. The amount and type of dietary fibre in the weaning diets of these rats influenced their fermentation capacity for these fibres as adult rats. Rats fed a particular dietary fibre at weaning had a greater fermentation capacity for that fibre as adults than rats who were fed the same fibre for the first time in adult life. Rats that were weaned onto a high fibre diet and then given a low fibre diet as adults produced more SCFA from the low fibre diet than rats who had been weaned onto it.

Studies of South African children under the age of three (Edwards *et al.*, 1998) showed that the fermentation capacity of black children was greater than that of white children. The black children produced significantly more SCFA, including butyrate, when faecal samples were fermented with a range of substrates using a model similar to that in this thesis. When the diets of the children were analysed, it was found that intakes of starch and fibre were similar. The black children however were weaned onto a diet of maize pap, which has been shown to contain 18g/100g resistant starch using the Englyst *in vitro* method. This suggests that it was the diet at weaning that affected their fermentation capacity at a later age which may have important implications for the risk of colon cancer and colitis, both of which are very rare in the black population.

Most studies of the development of infant flora have measured bacteria and their products in faeces. However, faecal SCFA may not reflect the true bacterial activity as they are the net result of both production and absorption and may not represent

events in the proximal colon. They give very little information on the ability of infants to ferment carbohydrate.

It is difficult to study colonic fermentation *in vivo*. *In vitro* studies using faecal bacteria to inoculate cultures have proved useful models of fermentation in adults (Adiotomre *et al.*, 1990). However, few studies of this sort have been carried out in infants. Those that have measured fermentation only of endogenous substrates or added sugars (Rasmussen *et al.*, 1988; Lifschitz *et al.*, 1990).

In a cross-sectional study of human infants (Parrett and Edwards, 1998, Chapter 2) the ability of infants to ferment simple and complex carbohydrates was tested. It was shown that breast-fed infants could ferment simple sugars equally at all stages of weaning, but the ability to ferment oligosaccharides did not develop until early weaning and the ability to ferment complex carbohydrates did not develop until late weaning. Complex carbohydrate fermentation did not reach the level of adults even at late weaning. In contrast, no statistical significant differences were seen in formula-fed infants between any stages of weaning for any substrate. The greater similarity of the colonic flora of formula-fed infants to that of the adult allows faster adaptation to complex carbohydrates. However, the capacity of formula-fed infants for complex carbohydrates at late weaning was still less than that of adults. This study was limited by the small numbers of infants and the cross-sectional nature of its design.

The lower ability of infants to ferment complex carbohydrates than adults suggests a slow maturation of the colonic flora and its fermentation capacity. This is further supported by studies indicating that key bacterial activities such as degradation of mucus, conversion of bilirubin to urobilinogen, of cholesterol to coprostanol and inactivation of faecal tryptic activity were still increasing at two years of age in Swedish children (Midvedt *et al.*, 1988, 1994; Midvedt and Midtvedt 1992). In addition starch was found in the faeces of children up to 3 years of age in the UK

suggesting an immature fermentation capacity for resistant starch (Verity and Edwards 1994).

In the study described in this chapter the fermentation capacity for simple and complex carbohydrates was investigated in breast-fed, formula-fed and mixed fed infants throughout the weaning process. Little information is available about fermentation capacity in infants. Although a cross-sectional pilot study had been undertaken (Chapter 2) this only used small numbers of infants. In order that more conclusive results could be drawn about fermentation capacity in infants it was considered necessary to investigate infants longitudinally. It has been suggested that bacterial activities develop slowly up to the age of two (Midtvedt *et al.*, 1988) and so infants in the study described here were followed through to two years of age. In the UK, although breast-feeding is encouraged, many mothers use formula milks. However, as mothers are encouraged to breast-feed many may start but then change to formula feeding either in part or fully, consequently many infants can be considered to be mixed fed (receiving a mixture of human and formula milk before weaning). It was important that this group was included in the study as very little information about mixed fed infants is available. Also more carbohydrates than in the pilot study were investigated to give an increased knowledge of fermentation capacity of different complex carbohydrates in infants.

## **7.2 METHODS**

### **7.2.1 Subjects**

Fermentation capacity was investigated in three different infant feeding groups, breast-fed ( $n = 28$ ), formula-fed ( $n = 16$ ) and mixed fed ( $n = 19$ ). Four development stages were investigated in each infant, 2 months (pre-weaning), four weeks after first giving any solid food (early weaning), 9 months (late weaning) and 18 months (very late weaning).

Infants were classified as breast-fed if they had been exclusively fed on breast milk until the introduction of solid foods. Before weaning the only addition they may have had to breast-milk was water. The formula-fed group were infants that had been exclusively formula-fed from birth. Infants were considered mixed fed if they had been breast-fed and then transferred completely to formula milk or if they received a mixture of breast and formula milk (Chapter 4, Table 4.2). Early weaning was defined as four weeks after giving solid food.

### **7.2.2 Sample collection and *in vitro* model**

A faecal sample was obtained at each development stage from each infant and this was used for the *in vitro* fermentation method (see Chapter 3 for full details). A faecal slurry (32% w/v) was made using Sorensens phosphate buffer (pH 7.0). One ml of faecal slurry was used to inoculate 9 mls of pre-reduced basic salts solution (Van Soest solution, Adiotomre *et al.*, 1990), containing 100 mg of carbohydrate substrate (Table 7.2).

Substrates were chosen to reflect a range of simple and complex carbohydrates (Table 7.2). Glucose is readily fermented by most bacteria and allows comparison of potential fermentation capacity more readily than do sugars or carbohydrates that need induction of enzymes. It is a major component sugar of many dietary fibres, resistant starch and maltodextrins. Raftilose™ was chosen as an example of a fructo-oligosaccharide, which may be present in significant quantities in breast milk. Soyabean polysaccharide (in soy flour) is used in weaning foods and is likely to reflect the sort of polysaccharides present in early and late weaning. These had all been used in the cross-sectional pilot study. In this present study lactose the main carbohydrate present in human milk, pectin and resistant starch were also included to study the relative fermentation capacity for different substrates in more detail.



<b>Table 7.2                      Description and source of substrates used</b>		
<b>Substrate</b>	<b>Description</b>	<b>Source</b>
Glucose	Easily fermented by all bacteria	Merck, Loughborough, UK
Lactose	Main carbohydrate source for infants	Sigma Chemical Company, Poole, UK
Raftilose™	Small quantities present in breast milk	Raffinerie tirllemontoise, SA
Soyabean polysaccharide	Often used in weaning foods	Scientific Hospital Supplies UK Ltd., Liverpool, UK
Pectin	Present in fruit which is often given at weaning	Merck, Loughborough, UK
Raw potato starch	Used as a model of resistant starch which may be produced in the processing of weaning foods	Rochet, France

The cultures were mixed thoroughly and gassed with oxygen free nitrogen before they were placed in an anaerobic jar. The jar was incubated at 37 °C for 24 hours. A control culture containing no carbohydrate substrate was set up and incubated in the same way.

After 24 hours, the universals were removed from the anaerobic jar and the pH of the fermentation fluid was measured. Culture samples were then stored at -20 °C for later analysis of SCFA and lactic acid.

Methods for sample collection and faecal short chain fatty acid assays are described in detail in Chapter 3.

### 7.2.3 Statistics

As there were three feeding groups, four development stages and six substrates as well as a control culture, the amount of data were considerable. After several discussions with several statisticians the following treatments were agreed. The data were not normally distributed. As there were four developmental stages to be

followed it was considered that longitudinal data were best compared by the one-sample Wilcoxon test. However, it was suggested that as few as possible Wilcoxon tests should be made on the data as a large amount of tests would increase the possibility of differences being significant by chance. Consequently only total SCFA and proportions of SCFA were tested. Because of the overall number of statistical tests this involves, more emphasis was put on statistical significance of  $p < 0.01$  rather than  $p < 0.05$ . Individual SCFA concentrations were not tested for significance although data for them are given.

For comparisons between the three feeding groups a non-parametric test (Kruskal-Wallis) was used as the data was not normally distributed. If by Kruskal-Wallis there was a significant difference this was considered to be a true difference and each group (breast, formula and mixed fed) could then be compared to each other.

### **7.3 RESULTS**

The feeding groups, development stages and large number of substrates produced many different results. To simplify the report of the results, these will be considered in sections as follows:

7.3.1 Longitudinal analysis of breast-fed infants

7.3.2 Longitudinal analysis of formula-fed infants

7.3.3 Longitudinal analysis of mixed fed infants

7.3.4 Comparison of breast-fed, formula-fed and mixed fed infants

Each section will then report the results for simple sugars, oligosaccharides, and complex carbohydrates.

### 7.3.1 Longitudinal analysis of breast-fed infants

#### 7.3.1.1 pH

The final pH was measured after 24-hour anaerobic culture of faecal samples from breast-fed infants with various carbohydrate sources (Table 7.3). These pH values have not been statistically compared as it was more appropriate to consider the decrease in pH from that of the control culture which standardises the data.

**Table 7.3** Final pH after 24-hour incubation of faecal samples from breast-fed infants (n=28) with various carbohydrates

	Pre-weaning	Early weaning	Late weaning (9 months)	Very late weaning (18 months)
Control	7.0 (6.3-7.9)	7.0 (6.0-7.6)	7.2 (6.5-7.6)	7.2 (6.4-7.8)
Glucose	4.8 (3.8-6.6)	5.0 (3.9-6.2)	5.4 (3.7-6.5)	5.4 (4.4-6.7)
Lactose	4.6 (3.7-6.3)	4.7 (3.9-5.6)	5.1 (4.0-6.2)	5.1 (4.0-6.5)
Raftilose™	4.8 (4.0-6.2)	4.9 (4.1-5.8)	5.1 (3.8-5.7)	5.2 (4.1-6.2)
Soyabean polysaccharide	6.7 (5.9-7.2)	6.7 (5.9-7.0)	6.7 (6.3-7.1)	6.6 (4.7-7.5)
Pectin	6.0 (4.6-6.7)	5.9 (4.7-6.9)	5.9 (4.7-6.6)	5.6 (4.8-6.6)
Starch	6.5 (5.1-7.1)	6.7 (5.1-7.3)	6.7 (5.7-7.1)	6.5 (5.5-7.0)

The final pH of cultures containing, glucose, lactose and raftilose™ increased in pH from pre-weaning onwards. The final pH values for soyabean polysaccharide, pectin and starch were similar to that of the control culture. This suggests that there is little fermentation occurring in these cultures. For soyabean polysaccharide and starch this remains the case throughout all weaning stages however, with pectin as a substrate, the final pH dropped by late weaning indicating that the ability to ferment pectin increased.

The decrease in pH after subtraction of the pH of the control culture is shown in Table 7.4.

#### **7.3.1.1.1 Simple sugars**

The decrease in pH from that of the control culture was less at late and very late weaning compared with pre-weaning with cultures of glucose ( $p < 0.05$ ).

A similar trend is seen with lactose with a lower decrease in pH throughout the development stages, however, this is only significant at very late weaning compared with pre-weaning ( $p < 0.05$ ).

#### **7.3.1.1.2 Oligosaccharide**

With raftilose™ although the same trend was seen as for simple sugars, there were no significant differences between any development stage.

#### **7.3.1.1.3 Complex carbohydrates**

With all complex carbohydrates, the pH fell from pre through to very late weaning. With starch, however, there were no significant differences between any development stage.

Pre-weaning, early weaning and late weaning decreases in pH were significantly higher ( $p < 0.05$ ) than at very late weaning with soyabean polysaccharide.

With pectin the decrease in pH at very late weaning was significantly higher than both pre and early weaning ( $p < 0.01$ ). There was significantly less of a decrease in pH when comparing pre- and early weaning ( $p < 0.05$ ) but a significantly greater decrease in pH between late and early weaning ( $p < 0.01$ ). This indicates that there is an increased ability to ferment these carbohydrates as weaning progresses but still did not achieve comparable pH drops as the sugars and oligosaccharide (Table 7.4).

**Table 7.4 Decrease in pH of cultures with various carbohydrates from culture with no carbohydrate after 24-hour incubation of faecal samples from breast-fed infants (n=28)**

	Pre-weaning	Early weaning	Late weaning (9 months)	Very late weaning (18 months)
Glucose	2.2	2.1	1.8 <sup>†</sup>	1.9 <sup>†</sup>
Lactose	2.4	2.3	2.0	1.8 <sup>†</sup>
Raftilose™	2.0	2.1	2.0	1.9
Soyabean polysaccharide	0.4	0.4	0.5	0.6 <sup>††</sup>
Pectin	1.1	1.0 <sup>*</sup>	1.2 <sup>††</sup>	1.6 <sup>**††</sup>
Starch	0.6	0.4	0.4	0.7

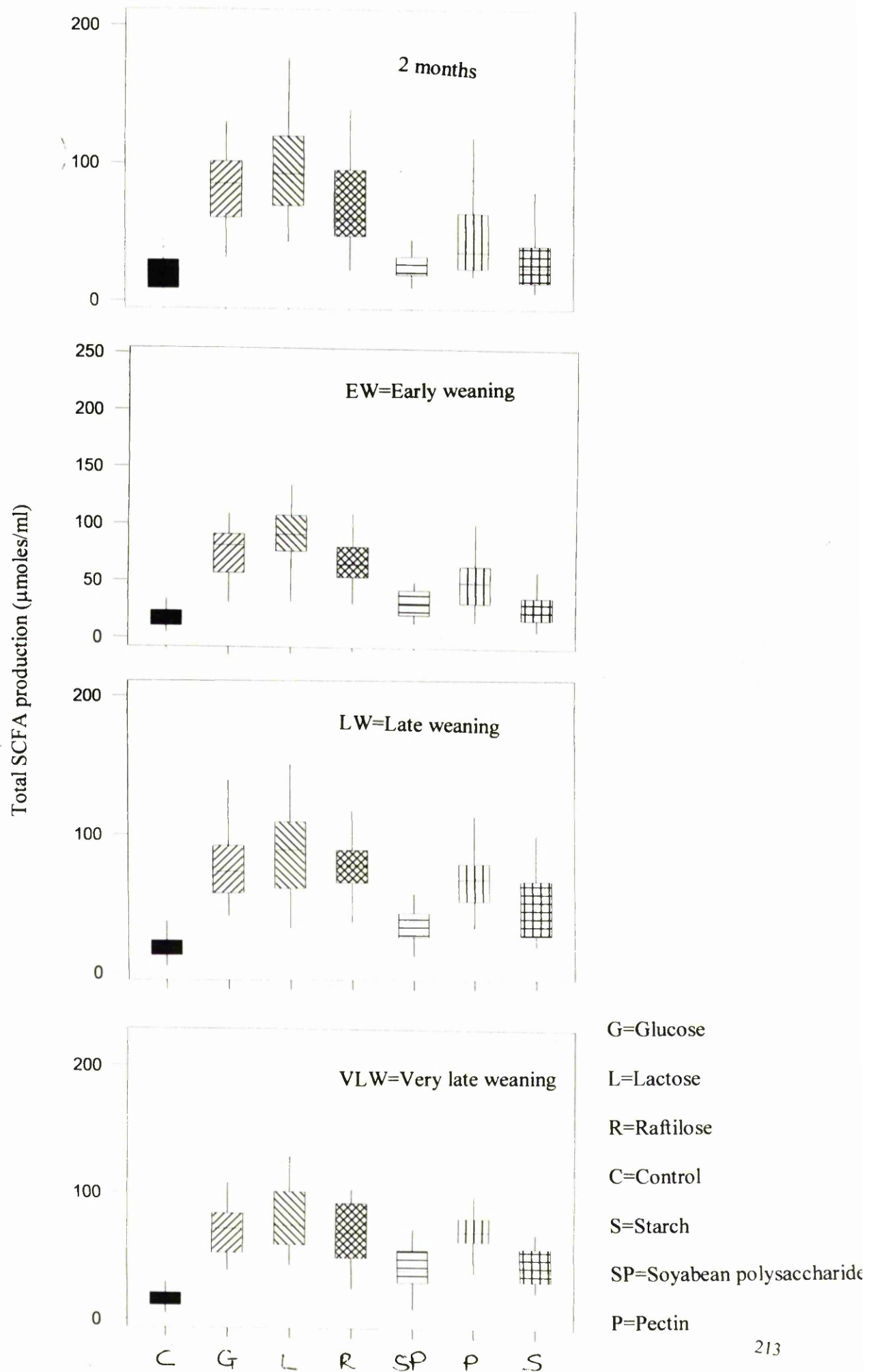
p<0.05, \*\* p<0.01 compared with pre-weaning, † p<0.05, †† p<0.01 compared with early weaning, ‡ p<0.05 compared with late weaning

### **7.3.1.2 Fermentation capacity measured by SCFA and lactate production of breast-fed infants**

Total SCFA from bacterial cultures of faeces from breast-fed infants with various carbohydrates were expressed after subtraction of results from the control culture (Table 7.5). For comparison Figure 7.1 shows the total SCFA from bacterial cultures of faeces from breast-fed infants with various carbohydrates without subtraction of results from the control culture. Subtraction of the SCFA produced by the control culture takes into account SCFA production from any endogenous carbon source.

#### **7.3.1.2.1 Simple sugars**

With cultures of glucose and lactose there was a gradual decrease in total SCFA produced between pre-weaning and very late weaning. This decrease was significantly different between very late weaning and pre-weaning (p<0.05 for glucose, p<0.01 for lactose).



#### **7.3.1.2.2 Oligosaccharide**

There was an increase in total SCFA produced with raftilose™ between early and late weaning ( $p < 0.05$ ). However given the large number of statistical tests performed results of significance  $p < 0.05$ , should be treated with caution.

#### **7.3.1.2.3 Complex carbohydrates**

With cultures of soyabean polysaccharide, pectin and starch far more statistically significant differences were seen between each weaning stage. Total SCFA production increased throughout weaning. Production of SCFA was significantly increased between pre-weaning and late and very late weaning for all three carbohydrates ( $p < 0.01$  for all except for starch between pre and very late weaning  $p < 0.05$ ). There was a significant increase in total SCFA between early and late and very late weaning for pectin and starch ( $p < 0.05$  early vs late, 0.001 early vs very late for pectin,  $p < 0.01$  early vs very late for starch). With soyabean polysaccharide, total SCFA were significantly higher at very late weaning than at early weaning ( $p < 0.01$ ) and late weaning ( $p < 0.05$ ).

#### **7.3.1.3 Total SCFA production without lactate**

Lactate may contribute substantially to total SCFA production especially at pre-weaning with breast-fed infants where it is one of the predominant SCFA produced. However in previous studies lactate was not measured therefore total SCFA was also analysed without lactate (Table 7.6). Most significant differences were similar to total SCFA including lactate, however differences between pre- and very late weaning with glucose, and between early and late weaning with raftilose™ have disappeared. This suggests that these differences were due to high amounts of lactic acid production in these cultures at the early development stages.

**Table 7.5 Total SCFA including lactate production (μmoles/ml) from faecal cultures of breast-fed infants (n=28) after subtraction of total SCFA and lactate production of control culture**

		Total SCFA including lactate (μmoles/ml)			
		Pre-weaning	Early Weaning	Late Weaning	Very Late Weaning
Glucose	Median	66.3	63.0	59.0	52.7*
	Range	22.6-162.7	0-89.8	0-189.1	27.8-92.5
Lactose	Median	76.5	75.3	71.2	61.1
	Range	25.9-186.8	0-235.1	0-138.7	26.8-101.8
Raftilose™	Median	45.2	48.7	59.8†	56.1
	Range	12.8-127.2	0-92.2	0-140.5	8.5-144.8
Soy polysaccharide	Median	10.0	12.9	19.9*	25.7***†††
	Range	0-28.57	0-33.34	0-44.19	3.40-48.07
Pectin	Median	18.61	32.26	50.94**†	53.07***†††
	Range	0-107.33	0-76.34	0-119.38	21.58-72.94
Starch	Median	7.33	8.05	16.40***†	27.33***††
	Range	0-50.50	0-61.99	0-78.48	6.13-200.17

\*p<0.05, \*\*p<0.01 compared with pre-weaning; † p<0.05, †† p<0.01, ††† p<0.001 compared with early weaning; ‡ p<0.05 compared with late weaning.

### 7.3.1.4 Individual SCFA concentrations

Individual SCFA concentrations are shown in Table 7.7. These have not been analysed statistically as it would mean too many analyses and significant differences would occur by chance (see 7.2.3).

#### 7.3.1.4.1 Simple sugars and oligosaccharide

Glucose, lactose and raftilose™ have a predominantly acetate and lactate profile at pre-weaning, however, even by early weaning lactate was decreasing at the expense of propionate. Butyrate was present at late weaning but it is not until very late weaning that it is present in similar amounts to propionate (Table 7.7).



**Table 7.6 Total SCFA production without lactate ( $\mu$ moles/ml) of faecal cultures of breast-fed infants (N=28) after subtraction of total SCFA from control culture**

		Total SCFA without lactate ( $\mu$ moles/ml)			
		Pre-weaning	Early Weaning	Late Weaning	Very Late weaning
Glucose	Median	43.6	45.3	42.7	47.6
	Range	0-98.5	0-73.3	0-98.2	0-78.2
Lactose	Median	48.3	51.8	59.9	51.3
	Range	15.37-100.7	0-235.0	0-112.8	1.5-94.4
Raftilose™	Median	33.9	39.0	53.5*	53.4*
	Range	11.8-113.3	0-74.1	0-113.1	3.6-144.8
Soy polysaccharide	Median	10.0	12.9	19.9**	25.7**†††‡
	Range	0-28.6	0-33.3	0-36.4	3.4-48.1
Pectin	Median	18.6	27.6	50.9***	52.7***
	Range	0-107.3	0-76.3	0-112.8	21.6-70.2
Starch	Median	7.3	8.1	16.4***	27.3***
	Range	0-50.5	0-62.0	0-78.5	5.2-200.2

\*p<0.05, \*\*p<0.01 compared with pre-weaning; † p<0.05, †† p<0.01, ††† p<0.001 compared with early weaning; ‡ p<0.05 compared with late weaning.

#### 7.3.1.4.2 Complex carbohydrates

With the complex carbohydrates even at pre- weaning there was no lactate produced and the SCFA profile was dominated by acetate and propionate. Butyrate production increased throughout weaning although there were negligible amounts before weaning (Table 7.7).

**Table 7.7 Individual SCFA concentrations ( $\mu$ moles/ml) produced in *in vitro* cultures of faecal bacteria in breast-fed infants (n=28)**

<b>Pre-weaning</b>	<b>Acetate</b>	<b>Propionate</b>	<b>n-Butyrate</b>	<b>Lactate</b>
Blank	9.1 (2.3-21.9)	3.1 (0.8-20.3)	0.5 (0.0-2.6)	0 (0.0-44.7)
Glucose	55.6 (6.2-108.2)	3.3 (0.0-14.8)	0.1 (0.0-16.3)	26.1 (0.0-105.3)
Lactose	55.8 (30.1-110.5)	4.3 (0.0-19.0)	0.0 (0.0-2.4)	26.7 (0.0-158.3)
Raftilose™	39.1 (18.1-113.1)	5.0 (0.0-24.1)	0.0 (0.0-14.6)	10.5 (0.0-60.4)
Soyabean polysaccharide	17.2 (6.4-34.5)	6.2 (1.8-28.9)	0.3 (0.0-4.6)	0.0 (0.0-24.5)
Pectin	29.1 (13.7-104.5)	6.6 (0.6-23.7)	0.0 (0.0-8.0)	0 (0.0-20.9)
Starch	17.0 (4.7-54.1)	3.5 (0.4-24.3)	0.6 (0.0-5.5)	0 (0.0-15.0)
<b>Early weaning</b>	<b>Acetate</b>	<b>Propionate</b>	<b>n-Butyrate</b>	<b>Lactate</b>
Blank	9.3 (3.5-41.4)	2.8 (0.3-32.5)	0.6 (0.0-26.1)	0 (0.0-9.2)
Glucose	51.2(14.5-70.0)	8.4 (0.5-34.7)	1.2 (0.0-19.5)	6.5 (0.0-57.7)
Lactose	60.3 (25.1-233.7)	7.8 (0.0-28.9)	0.2 (0.0-8.7)	16.5 (0.0-76.8)
Raftilose™	43.7 (3.9-76.9)	12.1(1.3-26.6)	0.5 (0.0-20.3)	0.0 (0.0-66.4)
Soyabean polysaccharide	19.9 (5.6-36.7)	5.6 (3.1-16.6)	1.2 (0.0-5.9)	0 (0.0-13.4)
Pectin	36.7 (9.3-72.5)	7.6 (0.7-20.2)	0.5 (0.0-10.9)	0 (0.0-24.6)
Starch	17.2 (5.9-52.4)	4.3 (0.4-23.6)	0.9 (0.0-18.6)	0 (0.0-2.9)
<b>Late weaning</b>	<b>Acetate</b>	<b>Propionate</b>	<b>n-Butyrate</b>	<b>Lactate</b>
Blank	8.1 (3.8-65.9)	2.4 (0.0-17.6)	1.7 (0.0-17.2)	0 (0.0-3.0)
Glucose	39.4 (17.8-117.4)	10.1 (0.0-41.8)	5.2 (0.0-18.5)	0.4 (0.0-112.2)
Lactose	54.7 (21.9-131.7)	8.3 (0.0-31.2)	3.0 (0.0-21.1)	0 (0.0-94.8)
Raftilose™	50.5 (19.3-130.6)	9.5 (0.0-27.6)	4.8 (0.0-23.8)	0 (0.0-54.1)
Soyabean polysaccharide	23.3 (6.9-34.4)	5.9 (0.9-12.4)	4.0 (0.0-11.2)	0 (0.0-12.9)
Pectin	49.7 (26.3-130.2)	9.7 (0.0-17.7)	4.1 (0.0-7.8)	0 (0.0-52.7)
Starch	22.9 (10.7-93.4)	4.9 (0.0-33.5)	5.5 (0.0-19.0)	0 (0.0-11.1)
<b>Very late weaning</b>	<b>Acetate</b>	<b>Propionate</b>	<b>n-Butyrate</b>	<b>Lactate</b>
Blank	9.4 (2.3-17.3)	1.9 (0.3-5.2)	2.3 (0.0-5.6)	0 (0.0-0.0)
Glucose	40.4 (10.6-73.0)	8.5 (0.0-36.1)	10.0 (0.0-26.8)	1.4 (0.0-50.4)
Lactose	44.4 (16.8-74.9)	6.8 (0.0-35.8)	7.8 (0.0-33.1)	3.9(0.0-44.4)
Raftilose™	46.2 (17.9-112.8)	11.0 (0.9-38.8)	10.6 (0.0-39.1)	0 (0.0-20.0)
Soyabean polysaccharide	26.2 (7.5-46.8)	6.3 (0.8-16.3)	4.8 (0.0-14.6)	0 (0.0-5.6)
Pectin	51.7 (30.4-73.9)	8.0 (2.6-17.4)	5.6 (0.0-15.4)	0 (0.0-27.0)
Starch	25.1 (15.1-143.6)	5. 0(1.2-39.5)	9.2 (0.0-24.7)	0 (0.0-5.29)

### 7.3.1.5 Proportions of SCFA

The proportions of individual SCFA are given in Table 7.8. Only the proportions of acetate, propionate, butyrate and lactate are given as these are the main SCFA and quantities of other SCFA were negligible.

**Table 7.8** Proportions of individual SCFA concentrations produced in *in vitro* cultures of faecal bacteria in breast-fed infants (n=28)

<b>Pre-weaning</b>	<b>Acetate</b>	<b>Propionate</b>	<b>n-Butyrate</b>	<b>Lactate</b>
Blank	64.6 (23.2-86.8)	22.3 (5.9-45.3)	2.0 (0.0-19.3)	0.0 (0.0-69.7)
Glucose	63.8 (14.6-99.1)	4.4 (0.1-27.6)	0.2 (0.0-21.1)	28.0 (0.0-84.8)
Lactose	62.6 (18.3-98.6)	4.9 (0.0-28.9)	0.1 (0.0-2.9)	32.2 (0.0-78.3)
Raftilose™	58.8 (4.3-113.1)	10.6 (0.0-39.3)	0.6 (0.0-25.2)	3.5 (0.0-79.0)
Soyabean polysaccharide	70.9 (33.0-89.5)	21.6 (9.1-44.2)	1.0 (0.0-14.3)	0.0 (0.0-54.2)
Pectin	74.6 (60.3-94.9)	18.4 (2.5-36.5)	0.0 (0.0-14.6)	0.0 (0.0-27.6)
Starch	72.7 (48.8-94.6)	18.1 (3.0-38.7)	1.4 (0.0-12.8)	0.0 (0.0-37.8)
<b>Early weaning</b>	<b>Acetate</b>	<b>Propionate</b>	<b>n-Butyrate</b>	<b>Lactate</b>
Blank	64.4 (23.9-92.2)	16.7 (7.3-33.2)*	4.1 (0.0-18.6)*	0.0 (0.0-27.1)
Glucose	62.7 (30.5-99.3)	13.4 (0.6-40.2)*	0.3 (0.0-21.4)*	8.8 (0.0-66.0)
Lactose	64.1 (33.9-96.2)	7.8 (0.0-31.02)	0.2 (0.0-21.1)	15.8 (0.0-64.8)
Raftilose™	43.7 (3.9-76.9)***	12.1 (1.3-26.6)***	0.5 (0.0-20.3)***	0.0 (0.0-66.4)**
Soyabean polysaccharide	67.0 (25.9-80.6)	20.3 (10.1-34.2)	3.4 (0.0-14.8)	0.0 (0.0-32.9)
Pectin	75.6 (49.4-90.9)	18.7 (3.3-41.7)	1.6 (0.0-13.5)*	0.0 (0.0-34.4)
Starch	71.9 (56.9-91.5)	18.5 (2.1-39.4)	3.1 (0.0-23.1)	0.0 (0.0-7.3)
<b>Late weaning</b>	<b>Acetate</b>	<b>Propionate</b>	<b>n-Butyrate</b>	<b>Lactate</b>
Blank	61.8 (28.6-92.5)	14.0 (0.0-26.8)**†	13.5 (0.0-39.8)***††	0.0 (0.0-20.9)
Glucose	59.6 (21.7-98.4)	12.5 (0.0-48.7)**	6.6 (0.0-45.8)***††	0.6 (0.0-73.8)*
Lactose	64.7 (19.5-98.3)	11.5 (0.0-35.0)*	4.7 (0.0-46.0)****††	0.0 (0.0-78.0)**
Raftilose™	69.5 (31.8-97.2)†††	12.9 (0.0-32.4)	7.2 (0.0-40.4)***†††	0.0 (0.0-57.6)
Soyabean polysaccharide	64.3 (43.6-83.4)	17.3 (5.2-28.7)***††	10.4 (0.0-24.4)***†††	0.0 (0.0-22.8)
Pectin	76.4 (47.8-89.9)	14.0 (0.0-27.1)†	6.8 (0.0-12.8)***†††	0.0 (0.0-42.6)
Starch	65.9 (34.9-93.7)	13.9 (0.0-51.1)††	13.6 (0.0-26.7)***†††	0.0 (0.0-20.4)
<b>Very late weaning</b>	<b>Acetate</b>	<b>Propionate</b>	<b>n-Butyrate</b>	<b>Lactate</b>
Blank	58.8 (45.3-85.3)	12.9 (3.2-23.8)***†††††	14.0 (0.0-35.4)***††	0.0 (0.0-0.0)
Glucose	57.3 (22.2-84.0)	12.7 (0.0-44.0)**	12.8 (0.0-42.9)***†††	2.1 (0.0-75.7)**
Lactose	59.1 (35.5-91.7)	9.6 (0.0-34.5)*	12.1 (0.0-43.1)****†††	5.6 (0.0-58.8)*
Raftilose™	66.6 (38.1-84.3)†††	12.3 (3.2-37.4)	15.0 (0.0-46.9)***†††	0.0 (0.0-21.7)
Soyabean polysaccharide	62.9 (39.5-92.6)	14.5 (6.4-37.1)*†	11.6 (0.0-24.3)***†††	0.8 (0.0-10.9)
Pectin	76.2 (44.7-91.6)	11.4 (5.2-27.4)††	8.0 (0.0-18.7)***†††	0.0 (0.0-38.9)
Starch	59.6 (42.5-89.1)†††	10.3 (4.2-28.4)††	18.8 (0.0-39.9)***††††	0.0 (0.0-16.8)

\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 compared with pre-weaning; † p < 0.05, †† p < 0.01, ††† p < 0.001 compared with early weaning; ‡ p < 0.05 compared with late weaning.

In general, proportions of acetate remained constant throughout the development stages. Propionate and butyrate increased as weaning progresses and the proportion of lactate fell. However lactate was not produced by many cultures containing

complex carbohydrates throughout weaning. In cultures containing raftilose™, acetate proportions increased between early, late and very late weaning ( $p < 0.001$ ). In contrast, in cultures with starch there was a significant reduction in acetate between early and very late weaning ( $p < 0.001$ ).

**Table 7.9 Differences in total SCFA production between substrates after *in vitro* cultures of faecal bacteria in breast-fed infants (n=28)**

<b>Pre-weaning</b>	Glucose	Lactose	Raftilose™	Soyabean polysaccharide	Pectin	Starch
Glucose	x	↓*	↑*	↑***	↑***	↑***
Lactose	↑*	x	↑*	↑***	↑***	↑***
Raftilose™	↓*	↓*	x	↑***	↑**	↑***
Soyabean polysaccharide	↓***	↓***	↓***	x	↓***	↑
Pectin	↓***	↓***	↓**	↑***	x	↑**
Starch	↓***	↓***	↓***	↓	↓**	x
<b>Early weaning</b>						
Glucose	x	↓**	↑*	↑***	↑***	↑***
Lactose	↑**	x	↑***	↑***	↑***	↑***
Raftilose™	↓*	↓***	x	↑***	↑**	↑***
Soyabean polysaccharide	↓***	↓***	↓***	x	↓***	↑
Pectin	↓***	↓***	↓**	↑***	x	↑**
Starch	↓***	↓***	↓***	↓	↓**	x
<b>Late weaning</b>						
Glucose	x	↓	↓	↑***	↑	↑***
Lactose	↑	x	↑	↑***	↑*	↑***
Raftilose™	↑	↓	x	↑***	↑*	↑***
Soyabean polysaccharide	↓***	↓***	↓***	x	↓***	↑
Pectin	↓	↓*	↓*	↑***	x	↑***
Starch	↓***	↓***	↓***	↓	↓***	x
<b>Very late weaning</b>						
Glucose	x	↓**	↓	↑***	↓	↑***
Lactose	↑**	x	↑**	↑***	↑	↑***
Raftilose™	↑	↓**	x	↑***	↑	↑**
Soyabean polysaccharide	↓***	↓***	↓***	x	↓***	↓
Pectin	↑	↓	↓	↑***	x	↑***
Starch	↓***	↓***	↓**	↑	↓***	x

Reading along the substrate in the row gives the increase or decrease from the substrate in the column and its significant difference, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

### **7.3.1.6 Differences between substrates**

The ability of the flora to ferment the different substrates differed significantly (Table 7.9). At pre- and early weaning, apart from soyabean polysaccharide and starch, all substrates were significantly different from each other.

At late weaning there were no significant differences between the SCFA produced from glucose, lactose and raftilose™ and no significant difference between soyabean polysaccharide and starch. By very late weaning glucose and raftilose™; glucose and pectin; and soyabean polysaccharide and starch produced similar amounts of total SCFA.

## **7.3.2 Longitudinal analysis of formula-fed infants**

### **7.3.2.1 pH**

The values for pH after 24-hour anaerobic culture of faecal samples from formula-fed infants with various carbohydrate sources are given in Table 7.10. As with breast-fed infants the simple sugars and oligosaccharide have lower final pH than the control culture from pre-weaning. Also the complex carbohydrates have similar final pH to that of the control culture, and again by very late weaning the final pH of pectin culture was lower than at the other stages of weaning suggesting an adaptation of the infants to ferment pectin.

**Table 7.10 Final pH values after 24-hour incubation of faecal samples from formula-fed infants (n=16) with various carbohydrates**

	Pre-weaning	Early weaning	Late weaning (9 months)	Very late weaning (18 months)
Blank	7.2 (6.7-7.6)	7.0 (6.7-7.6)	7.2 (6.8-7.5)	7.1 (6.6-7.7)
Glucose	5.2 (4.1-6.0)	4.7 (4.4-6.2)	5.1 (3.9-6.1)	5.3 (4.2-6.2)
Lactose	5.1 (4.1-5.9)	4.5 (4.0-6.2)	5.0 (4.1-6.4)	5.2 (3.9-6.2)
Raftilose™	5.1 (4.2-6.0)	4.6 (4.1-6.0)	5.0(4.5-6.0)	5.2 (4.2-6.1)
Soyabean polysaccharide	6.9 (6.5-7.2)	6.7 (6.0-7.1)	6.7 (5.8-7.2)	6.4 (6.0-6.9)
Pectin	6.3 (5.1-7.1)	5.9 (4.8-7.0)	5.6 (4.9-6.7)	5.6 (4.8-6.9)
Starch	6.7 (6.0-7.2)	6.6 (4.9-7.0)	6.5 (5.6-7.3)	6.5 (5.4-7.1)

Decreases in final pH of cultures after subtraction of the final pH of the control culture are given in Table 7.11.

#### **7.3.2.1.1 Simple sugars**

There were no significant differences between any development stage for either glucose or lactose. The trend for both was a lower decrease between early weaning and very late weaning.

#### **7.3.2.1.2 Oligosaccharides**

A similar trend was seen with raftilose™ as with glucose. The decrease in pH becomes higher between pre and late weaning and then falls at very late weaning. The decrease in pH at very late weaning was significantly lower than at early weaning ( $p < 0.05$ ).

### 7.3.2.1.3 Complex carbohydrates

With cultures containing soyabean polysaccharide and pectin the decrease in pH became greater from pre-weaning to very late weaning. The decrease was significantly greater at late ( $p < 0.05$  with soyabean polysaccharide;  $p < 0.01$  with pectin) and very late weaning ( $p < 0.05$ ) than at pre-weaning. Similar decreases in pH were seen at each development stage with starch but there were no significant differences.

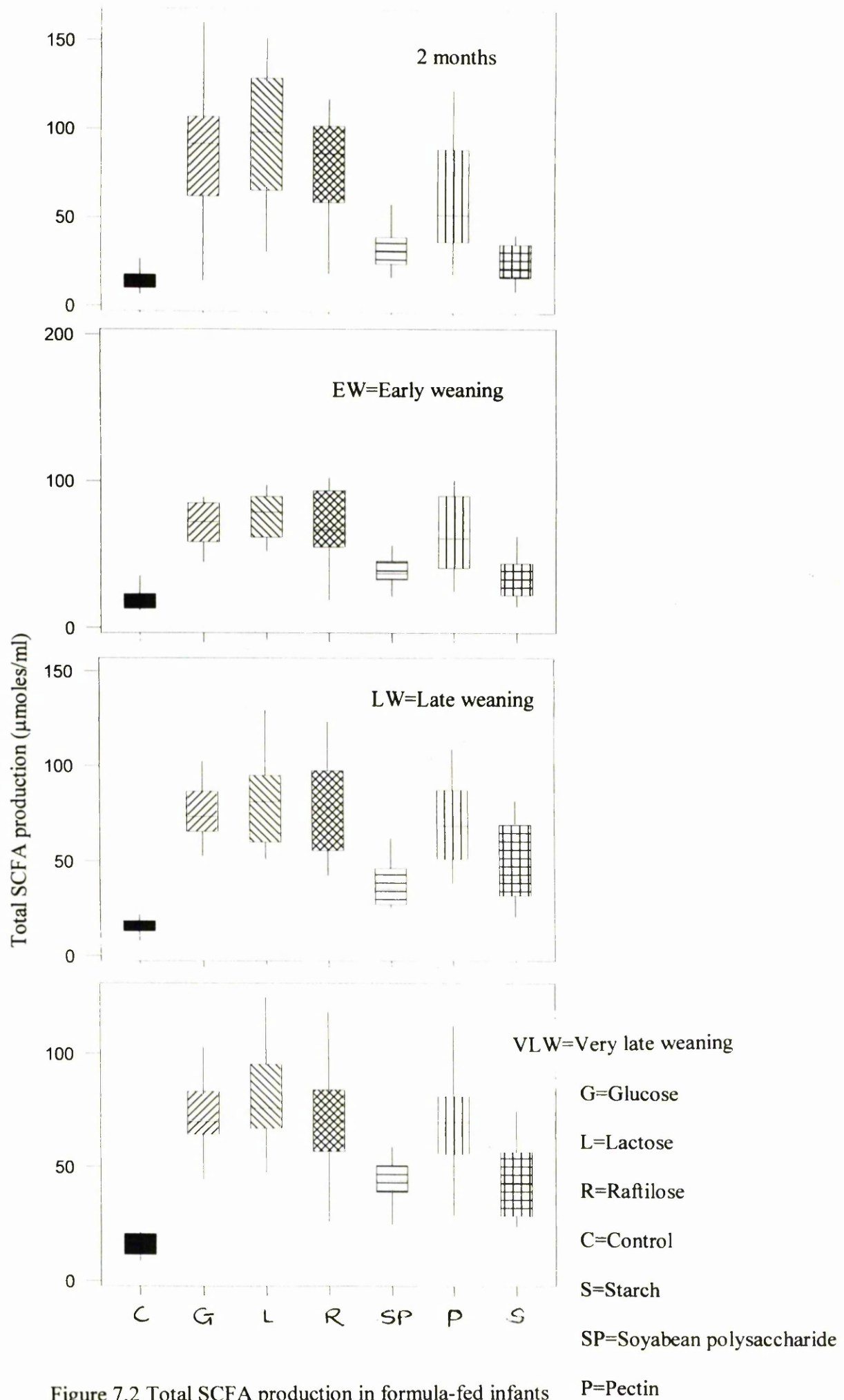
**Table 7.11** Decrease in pH of cultures with various carbohydrates from control culture after 24-hour incubation of faecal samples from formula-fed infants (n=16)

	Pre-weaning	Early weaning	Late weaning	Very late weaning
Glucose	1.89	2.11	2.14	1.61
Lactose	2.06	2.42	2.19	1.84
Raftilose™	1.91	2.24	2.06	1.88 <sup>†</sup>
Soyabean polysaccharide	0.36	0.47	0.48 <sup>*</sup>	0.56 <sup>*</sup>
Pectin	0.97	1.43	1.43 <sup>**</sup>	1.52 <sup>*</sup>
Starch	0.53	0.44	0.54	0.65

\* $p < 0.05$ , \*\*  $p < 0.01$  compared with pre-weaning, <sup>†</sup>  $p < 0.05$  compared with early weaning

### 7.3.2.2 Fermentation capacity measured by SCFA and lactate production of formula-fed infants

Total SCFA, including lactic acid, from bacterial cultures are shown after subtraction of results from the control cultures (Table 7.12). For comparison Figure 7.2 shows the total SCFA, including lactic acid, from bacterial cultures without subtraction of results from the control cultures. The only significant differences seen in the formula-fed infants' faecal bacterial cultures in production of total SCFA (including lactate) were between pre- and very late weaning with raftilose™ ( $p < 0.05$ ), and between pre- and late weaning with starch ( $p < 0.05$ ).





**Table 7.12**      **Total SCFA including lactate production (μmoles/ml) from faecal cultures of formula-fed infants (n=16) after subtraction of total SCFA and lactate production of control culture**

		Total SCFA including lactate (μmoles/ml)			
		Pre-weaning	Early Weaning	Late Weaning	Very Late weaning
G	Median	75.4	52.1	62.4	52.7
	Range	0-146.2	27.6-134.1	30.1-104.4	26.8-93.5
L	Median	83.1	54.8	64.5	65.5
	Range	21.9-137.8	35.6-153.9	24.6-137.4	26.0-103.4
R	Median	69.5	44.5	59.8	55.9*
	Range	10.1-103.5	3.7-163.6	28.6-107.7	0-82.8
SP	Median	15.0	20.2	20.1	23.5
	Range	0.0-41.7	4.7-71.8	10.7-43.5	10.9-41.7
P	Median	41.9	48.0	46.3	50.2
	Range	0.0-108.9	0.0-168.1	23.2-83.1	0.0-95.1
S	Median	7.13	12.0	21.3*	25.4
	Range	0.0-58.77	0.0-75.3	13.0-66.5	0.0-66.3

\* p<0.05 compared with pre-weaning

G = Glucose, L = Lactose, R = Raftilose™, SP = Soy polysaccharide, P = Pectin, S = Starch

### 7.3.2.3 Total SCFA production without lactate

When total SCFA were compared without including lactate, (Table 7.13) differences seen previously with raftilose™ had disappeared. The increase of starch from pre- to late weaning became more marked ( $p < 0.01$ ) and there was also a significant increase between pre- and very late weaning ( $p < 0.05$ ). Cultures at very late weaning with soyabean polysaccharide were significantly higher than pre-, early and late weaning ( $p < 0.01$ ).

**Table 7.13 Total SCFA production without lactate (μmoles/ml) in faecal cultures of formula-fed infants (n=16) after subtraction of total SCFA from control culture**

		Total SCFA without lactate (μmoles/ml)			
		Pre-weaning	Early Weaning	Late Weaning	Very Late weaning
G	Median	49.9	39.3	47.0	47.9
	Range	0-94.0	18.8-121.9	19.3-89.7	23.6-61.9
L	Median	58.8	39.9	48.0	49.1
	Range	20.5-105.1	18.1-79.9	29.3-137.4	22.4-81.1
R	Median	48.2	43.0	51.0	55.0
	Range	10.1-86.2	2.6-163.6	15.6-99.5	0-63.4
SP	Median	15.0	19.9	18.9	23.5**††‡
	Range	0-32.6	2.7-38.2	3.8-43.5	10.9-41.7
P	Median	37.2	47.5	44.1	50.2
	Range	0.0-8.8	0.0-72.9	4.0-83.1	0.0-95.1
S	Median	5.7	12.0	18.7**	22.7*
	Range	0.0-47.8	0.0-75.3	12.2-63.9	0.0-66.3

\*p<0.05, \*\*p<0.01 compared with pre-weaning; †† p<0.01 compared with early weaning; ‡ p<0.01 compared with late weaning.

G = Glucose, L = Lactose, R = Raftilose™, SP = Soy polysaccharide, P = Pectin, S = Starch

### 7.3.2.4 Individual SCFA production

Concentrations of individual SCFA are given in Table 7.14. The amounts of SCFA produced seem to be dependent on substrate and there were few changes through the weaning stages.

#### 7.3.2.4.1 Simple sugars and oligosaccharide

With glucose, lactose and raftilose™, acetate was the main SCFA produced but propionate, butyrate and lactate were all present. Lactate decreased in late and very late weaning as butyrate increased.

### 7.3.2.4.2 Complex carbohydrates

With cultures of soyabean polysaccharide, pectin and starch, no lactate was produced at any stage of weaning. Acetate was the predominant SCFA, although propionate and butyrate were both present. Butyrate increased from pre-weaning through all stages of weaning.

**Table 7.14 Individual SCFA concentrations ( $\mu$ moles/ml) produced in *in vitro* cultures of faecal bacteria in formula-fed infants (n=16)**

<b>Pre-weaning</b>	<b>Acetate</b>	<b>Propionate</b>	<b>n-Butyrate</b>	<b>Lactate</b>
Blank	8.6 (4.0-38.5)	2.3 (0.6-5.7)	0.6 (0.0-5.1)	0 (0.0-3.2)
Glucose	43.8 (9.4-79.3)	14.7 (1.2-35.6)	0.9 (0.0-17.6)	9.8 (0.0-99.2)
Lactose	58.1 (12.7-96.4)	12.8 (2.2-37.3)	1.2 (0.0-17.4)	16.3 (0.0-79.1)
Raftilose™	40.9 (10.4-70.0)	13.9 (3.0-39.5)	1.7 (0.0-14.6)	11.3 (0.0-68.2)
Soyabean polysaccharide	19.7 (11.0-34.0)	7.5 (2.1-11.4)	1.1 (0.0-4.0)	0 (0.0-12.4)
Pectin	38.8 (11.7-64.3)	11.3 (2.5-24.3)	1.1 (0.0-3.7)	0 (0.0-51.5)
Starch	14.7 (4.8-43.2)	4.4 (0.6-7.7)	1.4 (0.0-9.6)	0 (0.0-29.6)
<b>Early weaning</b>	<b>Acetate</b>	<b>Propionate</b>	<b>n-Butyrate</b>	<b>Lactate</b>
Blank	9.4 (6.9-21.7)	2.3 (1.2-8.5)	1.8 (0.0-4.0)	0 (0.0-6.4)
Glucose	41.8 (12.9-59.8)	11.2 (0.8-77.9)	3.3 (0.0-17.4)	10.3 (0.0-74.9)
Lactose	45.3 (31.6-78.4)	8.6 (0.7-27.1)	2.9 (0.0-18.4)	12.9 (0.0-91.1)
Raftilose™	42.9 (15.8-66.7)	10.0 (1.0-106.9)	3.8 (0.0-15.3)	2.5 (0.0-79.8)
Soyabean polysaccharide	25.4 (13.5-35.1)	6.5 (2.9-12.2)	2.6 (0.3-15.5)	0 (0.0-34.6)
Pectin	42.3 (14.4-71.0)	11.7 (3.0-22.0)	2.5 (0.3-9.3)	0 (0.0-95.2)
Starch	16.9 (9.8-71.2)	5.0 (2.3-12.3)	2.93 (0.3-13.4)	0 (0.0-32.6)
<b>Late weaning</b>	<b>Acetate</b>	<b>Propionate</b>	<b>n-Butyrate</b>	<b>Lactate</b>
Blank	8.7 (4.1-16.0)	2.2 (0.8-4.9)	1.8 (0.0-5.0)	0 (0.0-12.9)
Glucose	45.7 (18.5-74.4)	7.5 (0.6-44.6)	4.9 (0.2-28.7)	9.8 (0.0-61.1)
Lactose	45.7 (23.6-95.1)	6.4 (0.9-66.1)	6.7 (0.4-27.2)	4.2 (0.0-54.0)
Raftilose™	42.2 (20.0-86.7)	7.8 (0.6-33.8)	9.2 (0.0-27.4)	3.1 (0.0-73.7)
Soyabean polysaccharide	21.1 (12.4-38.4)	5.2 (3.0-10.6)	3.5 (1.8-14.1)	0 (0.0-16.7)
Pectin	49.5 (15.2-76.5)	6.89 (3.0-17.6)	4.5 (0.6-13.3)	0 (0.0-47.7)
Starch	21.4 (10.9-60.2)	6.1 (1.9-33.2)	6.8 (1.1-30.8)	0 (0.0-11.9)
<b>Very late weaning</b>	<b>Acetate</b>	<b>Propionate</b>	<b>n-Butyrate</b>	<b>Lactate</b>
Blank	8.7 (6.1-24.3)	2.4 (1.4-5.4)	2.2 (0.0-7.3)	0 (0.0-0.0)
Glucose	40.2 (19.4-62.7)	9.3 (0.0-22.1)	8.7 (0.0-27.7)	3.2 (0.0-52.3)
Lactose	47.1 (22.0-64.8)	8.8 (0.0-24.3)	13.2 (0.0-24.2)	3.2 (0.0-54.5)
Raftilose™	42.9 (18.9-69.1)	9.1 (2.3-18.9)	11.8 (0.0-25.8)	0.9 (0.0-24.5)
Soyabean polysaccharide	26.6 (15.8-42.1)	7.4 (4.6-10.2)	5.4 (0.8-10.50)	0 (0.0-7.2)
Pectin	52.3 (16.3-83.3)	9.6 (3.6-21.6)	7.0 (0.0-12.2)	0 (0.0-6.4)
Starch	21.0 (14.7-52.5)	5.2 (3.7-9.0)	8.6 (0.14-24.8)	0 (0.0-11.5)

### 7.3.2.5 Proportions of individual SCFA

**Table 7.15** Proportions of individual SCFA concentrations in *in vitro* cultures of faecal bacteria in formula-fed infants (n=16)

Pre-weaning	Acetate	Propionate	n-Butyrate	Lactate
Blank	62.2 (42.2-77.0)	13.3 (4.0-27.2)	6.1 (0.0-15.6)	0.0 (0.0-22.0)
Glucose	56.3 (29.5-71.5)	18.3 (2.0-45.4)	1.1 (0.0-20.6)	11.9 (0.0-66.4)
Lactose	58.6 (26.2-79.4)	15.9 (1.6-51.7)	1.0 (0.0-21.6)	19.9 (0.0-59.4)
Raftilose™	54.9 (22.8-76.7)	23.6 (3.8-47.5)	3.2 (0.0-22.0)	14.8 (0.0-67.8)
Soyabean polysaccharide	58.9 (48.2-74.0)	24.0 (6.4-38.5)	4.3 (0.0-12.1)	0.0 (0.0-22.5)
Pectin	69.1 (44.0-81.1)	22.8 (7.0-38.3)	2.4 (0.0-7.4)	0.0 (0.0-41.9)
Starch	62.9 (28.7-78.2)	16.6 (3.0-31.9)	8.5 (0.0-18.8)	0.0 (0.0-40.7)
Early weaning	Acetate	Propionate	n-Butyrate	Lactate
Blank	58.5 (42.9-70.7)	14.9 (7.2-26.5)	11.2 (0.0-20.6)*	0.0 (0.0-22.2)
Glucose	54.8 (24.2-82.7)	16.9 (1.2-58.3)	3.4 (0.0-22.6)	15.7 (0.0-50.74)
Lactose	61.4 (36.9-85.6)	9.9 (1.2-32.1)*	2.6 (0.0-21.6)	19.0 (0.0-54.4)
Raftilose™	57.2 (38.0-86.1)	12.8 (3.3-60.9)	4.9 (0.0-31.4)	5.9 (0.0-50.8)
Soyabean polysaccharide	59.5 (41.2-77.9)	19.4 (5.4-28.4)*	2.4 (0.9-30.7)*	0.0 (0.0-40.6)
Pectin	69.8 (38.1-83.5)	18.2 (4.7-28.8)	4.7 (0.5-16.8))	0.0 (0.0-52.4)
Starch	62.5 (36.7-81.1)	15.2 (3.6-25.7)	10.3 (1.9-32.6))	0.0 (0.0-41.1)
Late weaning	Acetate	Propionate	n-Butyrate	Lactate
Blank	55.9 (32.2-73.5)	14.3 (4.2-23.1)	12.8 (0.0-17.1)**	0.0 (0.0-44.5)
Glucose	63.0 (31.3-86.2)	10.1 (0.7-43.6)	6.1 (0.2-48.5)*	16.1 (0.0-59.8)
Lactose	61.7 (44.0-80.3)	9.2 (0.9-44.0)	6.5 (0.4-50.7)**†	5.4 (0.0-46.6)
Raftilose™	62.3 (28.7-76.6)	11.6 (0.5-38.9)*	11.1 (0.0-45.3)*†	4.7 (0.0-66.7)
Soyabean polysaccharide	60.4 (21.3-85.2)	15.5 (6.7-25.2)*	10.2 (4.2-22.7)**	0.0 (0.0-47.3)
Pectin	74.6 (40.0-85.4)	12.9 (2.8-27.3)*†	6.6 (0.8-18.4)**	0.0 (0.0-50.6)
Starch	59.3 (28.1-77.1)	12.2 (4.2-51.3)	13.2 (2.8-56.1)*	0.0 (0.0-30.5)
Very late weaning	Acetate	Propionate	n-Butyrate	Lactate
Blank	55.1 (47.1-76.1)	14.5 (9.6-26.1)	12.2 (0.0-20.5)*	0.0 (0.0-0.0)
Glucose	55.4 (27.8-73.8)	14.1 (0.0-31.6)	14.3 (0.0-39.6)*†	4.8 (0.0-50.8)
Lactose	58.7 (31.1-75.4)	12.9 (0.0-34.3)	14.5 (0.0-34.2)*†	4.8 (0.0-44.7)
Raftilose™	59.4 (36.5-76.9)	12.7 (5.6-26.6)	17.2 (0.0-36.3)*†	1.3 (0.0-26.9)*
Soyabean polysaccharide	60.9 (50.2-73.3)	14.9 (10.9-30.5)*	12.2 (3.1-19.7)**	0.0 (0.0-12.3)
Pectin	72.8 (57.1-81.7)	13.9 (6.4-28.5)	11.5 (0.0-19.5)**††	0.0 (0.0-10.7)
Starch	58.5 (41.3-77.7)	13.4 (6.7-25.9)	17.0 (0.6-38.1)*†	0.0 (0.0-22.6)

\*p<0.05, \*\*p<0.01 compared with pre-weaning; †p<0.05 compared with early weaning; ‡ p<0.05 compared with late weaning.

No significant differences were seen for proportions of acetate for any substrate at any development stage. With cultures containing raftilose™ there was a significant

decrease in lactate from pre-to very late weaning ( $p < 0.05$ ). In general propionate increased from pre- to early weaning but these differences are less marked than in breast-fed infants. The biggest change was in butyrate which increased as weaning progressed.

**Table 7.16 Differences in total SCFA and lactate production ( $\mu\text{moles/ml}$ ) produced in *in vitro* cultures of faecal bacteria in formula-fed infants (n=16)**

<b>Pre-weaning</b>	Glucose	Lactose	Raftilose™	Soyabean polysaccharide	Pectin	Starch
Glucose	X	↓	↑	↑**	↑**	↑**
Lactose	↑	X	↑*	↑***	↑**	↑***
Raftilose™	↓	↓*	X	↑**	↑*	↑***
Soyabean polysaccharide	↓**	↓***	↓**	X	↓**	↑
Pectin	↓**	↓**	↓*	↑**	X	↑**
Starch	↓**	↓***	↓***	↓	↓**	X
<b>Early weaning</b>						
Glucose	X	↓	↑	↑***	↑	↑**
Lactose	↑	X	↑	↑***	↑	↑**
Raftilose™	↓	↓	X	↑**	↓	↑**
Soyabean polysaccharide	↓***	↓***	↓**	X	↓**	↑
Pectin	↓	↓	↑	↑**	X	↑**
Starch	↓**	↓**	↓**	↓	↓**	X
<b>Late weaning</b>						
Glucose	X	↓	↑	↑	↑	↑**
Lactose	↑	X	↑	↑***	↑*	↑**
Raftilose™	↓	↓	X	↑***	↑	↑**
Soyabean polysaccharide	↓***	↓***	↓***	X	↓***	↓
Pectin	↓	↓*	↓	↑***	X	↑*
Starch	↓**	↓**	↓**	↑	↓*	X
<b>Very late weaning</b>						
Glucose		↓*	↓	↑**	↑	↑**
Lactose	↑*		↑*	↑**	↑*	↑**
Raftilose™	↑	↓*		↑**	↑	↑*
Soyabean polysaccharide	↓**	↓**	↓**		↓**	↓
Pectin	↓	↓*	↓	↑**		↑*
Starch	↓***	↓**	↓***	↑***	↓*	

Reading along the substrate in the row gives the increase or decrease from the substrate in the column and its significant difference, \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

### **7.3.2.6 Differences between substrates**

At pre-weaning, glucose produced similar total SCFA to both lactose and raftilose™, whereas starch produced similar amounts to soyabean polysaccharide (Table 7.16). At early and late weaning these similarities remained. Also lactose was not significantly different to raftilose™ and pectin was not significantly different to glucose, lactose or raftilose™. Whilst at late weaning lactose became significantly different to glucose, raftilose™ and pectin.

There were less differences seen in the total amount of SCFA and lactate produced with different substrates at all development stages in formula-fed infants than in breast-fed infants. However, as with breast-fed infants, soy polysaccharide and pectin tended to produce much lower amounts of SCFA and lactate.

### **7.3.3 Longitudinal analysis of mixed fed infants**

#### **7.3.3.1 pH**

The values for final pH after 24-hour anaerobic culture of faecal samples from mixed fed infants with various carbohydrate sources are given in Table 7.17. The actual values of pH show that there was more fermentation with glucose, lactose and raftilose™ at all stages of weaning than with complex carbohydrate. Results for pectin showed that more fermentation was taking place as weaning progressed but soyabean polysaccharide and starch gave similar pH values at very late weaning as at pre-weaning.

**Table 7.17 Final pH values after 24-hour incubation of faecal samples from mixed fed infants (n=19) with various carbohydrates**

	Pre-weaning	Early weaning	Late weaning (9 months)	Very late weaning (18 months)
Blank	7.2 (6.7-7.7)	7.2 (7.0-7.5)	7.2 (6.7-7.7)	7.1 (6.7-7.6)
Glucose	4.9 (4.2-5.7)	5.4 (4.5-6.2)	5.2 (3.6-6.3)	4.8 (3.9-6.5)
Lactose	4.7 (4.1-5.7)	5.1 (4.5-6.1)	5.3 (3.7-6.4)	4.1 (3.9-6.5)
Raftilose™	5.2 (4.4-6.2)	5.2 (4.5-5.9)	5.3 (3.9-6.3)	5.0 (4.3-6.0)
Soyabean polysaccharide	7.0 (6.4-7.4)	6.9 (6.6-7.1)	6.8 (6.3-7.2)	6.6 (6.1-7.0)
Pectin	6.1 (5.0-6.9)	5.9 (5.1-6.7)	5.9 (5.0-6.6)	5.4 (4.7-7.0)
Starch	6.7 (5.8-7.4)	6.6 (5.2-7.0)	6.7 (4.7-7.4)	6.4 (5.3-7.1)

The decreases in pH of cultures from the control culture pH are shown in Table 7.18. Both glucose and lactose showed less of a decrease at early weaning than at late weaning ( $p < 0.01$ ,  $p < 0.05$  respectively). Cultures with soyabean polysaccharide had a higher decrease in pH at very late weaning than at pre- ( $p < 0.01$ ) and early ( $p < 0.05$ ) weaning suggesting that more fermentation is occurring at very late weaning than at the earlier development stages.

**Table 7.18 Decrease in pH of cultures with various carbohydrates from control culture after 24-hour incubation of faecal samples from mixed fed infants (n=19)**

	Pre-weaning	Early weaning	Late weaning (9 months)	Very late weaning (18 months)
Glucose	2.24	1.84**	1.91	2.31
Lactose	2.37	2.10*	2.12	2.46
Raftilose™	2.23	2.00	2.03	2.38
Soyabean polysaccharide	0.27	0.40	0.39	0.54***†
Pectin	1.02	1.40	1.34	1.62†
Starch	0.47	0.59	0.50	0.70

\* $p < 0.05$ , \*\*  $p < 0.01$  compared with pre-weaning, †  $p < 0.05$  compared with early weaning

### **7.3.3.2 Fermentation capacity measured by SCFA and lactate production of mixed fed infants**

When total SCFA including lactate were compared in bacterial cultures from faeces of mixed fed infants after subtraction of total SCFA and lactate from control cultures (Table 7.19) very few significant differences were seen between the development stages. For comparison Figure 7.3 shows the total SCFA including lactate in bacterial cultures from faeces of mixed fed infants without of total SCFA and lactate from control cultures.

#### **7.3.3.2.1 Simple sugars**

There were no significant difference in total SCFA and lactate with cultures of glucose and lactose. With both substrates more SCFA were produced at pre-weaning than at any of the other development stages but this did not reach significance. This indicates the mixed fed infants were able to ferment the simple sugars equally well at all development stages (Table 7.19).

#### **7.3.3.2.2 Oligosaccharide**

Again with the oligosaccharide there were no significant differences between any of the development stages. The amount of total SCFA and lactate produced was similar throughout (Table 7.19).

#### **7.3.3.2.3 Complex carbohydrates**

With cultures of soyabean polysaccharide and starch quite low amounts of total SCFA and lactate were initially produced. These increased through the development stages. Amounts at early weaning were significantly different from pre-weaning for both substrates ( $p < 0.05$ ,  $0.01$  respectively) and late weaning was significantly



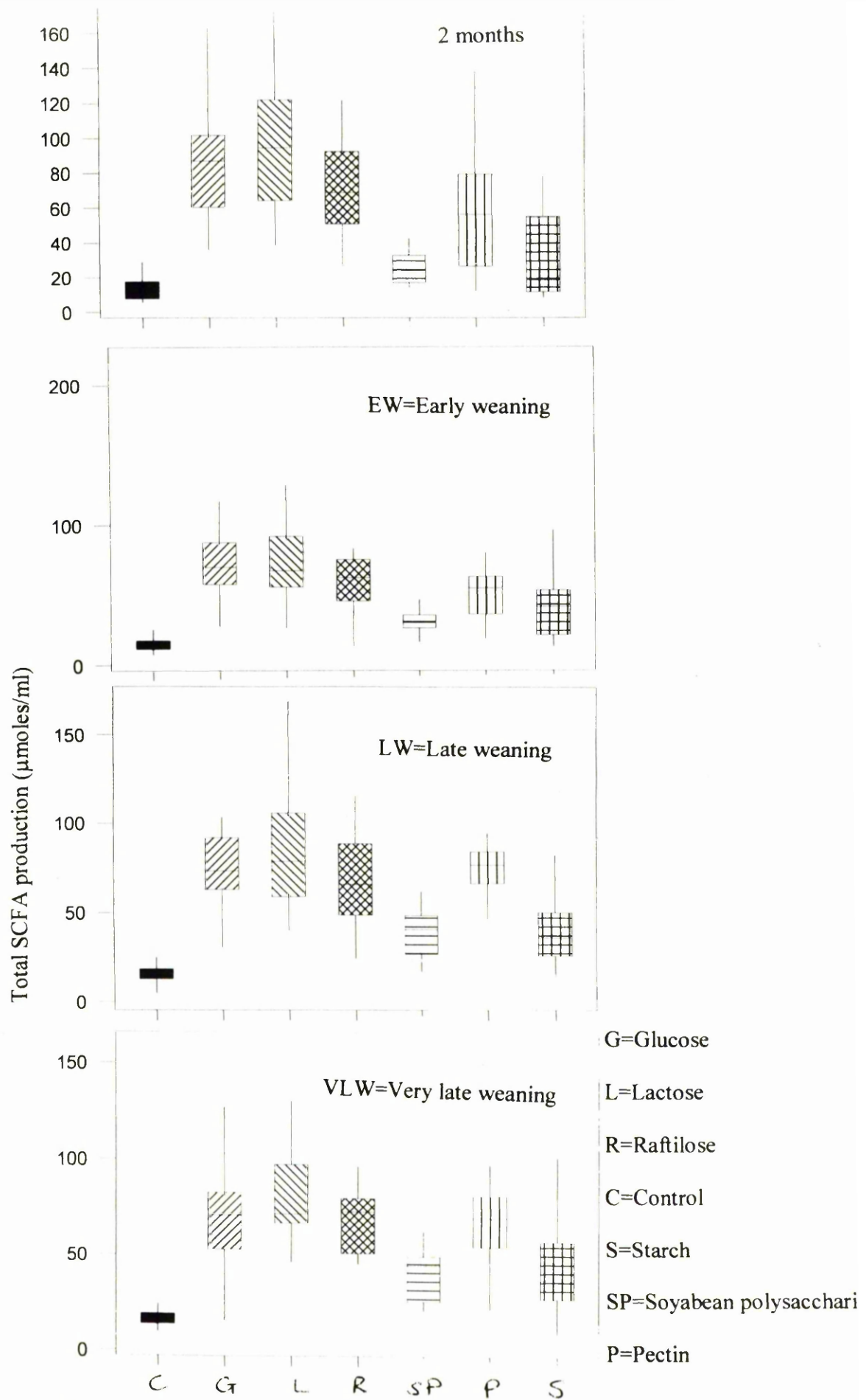


Figure 7.3 Total SCFA production in mixed fed infants

different from pre-weaning with soyabean polysaccharide ( $p < 0.05$ ). In cultures containing pectin, total SCFA and lactate increased from pre-weaning to late weaning but then decreased again at very late weaning, however, both late and very late weaning were significantly higher than early weaning ( $p < 0.01$ ).

**Table 7.19 Total SCFA including lactate production ( $\mu\text{moles/ml}$ ) in faecal cultures of mixed fed infants (n=19) after subtraction of total SCFA and lactate production of control culture**

		Total SCFA including lactate production ( $\mu\text{moles/ml}$ )			
		Pre-weaning	Early Weaning	Late Weaning	Very Late Weaning
Glucose	Median	67.8	55.3	59.5	55.4
	Range	28.5-147.3	12.7-148.8	0.0-115.7	0.0-142.2
Lactose	Median	76.7	54.8	63.1	57.6*
	Range	31.3-158.4	11.5-204.1	0.0-137.8	34.0-141.7
Raffilose <sup>TM</sup>	Median	50.9	48.7	50.7	51.3
	Range	21.4-105.9	0.0-124.0	0.0-134.6	29.5-76.6
Soy polysaccharide	Median	11.8	17.3*	18.3*	25.2
	Range	0.0-29.0	8.5-36.1	0.0-67.6	0.0-72.6
Pectin	Median	47.9	40.10	62.8 <sup>††</sup>	55.1 <sup>††</sup>
	Range	0.0-110.4	5.7-61.3	0.0-110.4	9.9-80.9
Starch	Median	6.0	27.1 <sup>**</sup>	21.1	26.7
	Range	0.0-49.1	4.4-85.2	0.0-9.7	0.0-85.2

$p < 0.05$ ,  $**p < 0.01$  compared with pre-weaning;  $†† p < 0.01$  compared with early weaning.

### 7.3.3.3 Total SCFA production without inclusion of lactate

Comparisons of total SCFA without inclusion of lactate are shown in Table 7.20. Differences seen when lactate was included all remained. In addition, cultures with pectin had higher total SCFA at late weaning than at pre-weaning ( $p < 0.05$ ). In cultures with starch the increase in SCFA between pre- and early weaning remained at late and very late weaning ( $p < 0.05$ ).

**Table 7.20** Total SCFA production without lactate ( $\mu\text{moles/ml}$ ) in faecal cultures of mixed fed infants (n=19) after subtraction of total SCFA without lactate from control cultures

		Total SCFA without lactate ( $\mu\text{moles/ml}$ )			
		Pre-weaning	Early Weaning	Late Weaning	Very Late weaning
Glucose	Median	46.25	49.42	49.09	38.58
	Range	25.35-106.62	12.70-92.96	0-115.71	0-78.84
Lactose	Median	50.84	52.79	46.71	46.47
	Range	31.34-113.73	11.46-166.65	0-137.79	29.29-80.24
Raftilose™	Median	37.80	42.22	48.15	45.94
	Range	21.35-96.61	0-74.35	0-134.55	24.83-67.72
Soy polysaccharide	Median	11.80	16.45*	18.25*	22.44
	Range	0-29.03	8.54-36.09	0-64.97	0-48.11
Pectin	Median	43.54	40.10	57.68**††	52.17†
	Range	0-70.42	5.68-61.23	0-110.44	9.93-80.93
Starch	Median	5.96	27.13**	18.39*	26.72*
	Range	0-48.36	4.42-83.26	0-89.65	0-85.19

\*p<0.05, \*\*p<0.01 compared with pre-weaning; † p<0.05, †† p<0.01 compared with early weaning.

#### 7.3.3.4 Individual SCFA concentrations

Individual SCFA are shown in Table 7.21, again these have not been statistically analysed as increase the statistical differences occurring by chance (see section 7.2.3).

In general acetate was the main SCFA for all substrates. Lactate was mainly produced by the simple sugars and raftilose™ at pre-weaning although glucose also produced lactate at all other development stages. Propionate concentrations tended to fall at early weaning as butyrate levels increased.

**Table 7.21 Individual SCFA concentrations (μmoles/ml) produced in *in vitro* cultures of faecal bacteria in mixed fed infants (n=19)**

<b>Pre-weaning</b>	<b>Acetate</b>	<b>Propionate</b>	<b>n-Butyrate</b>	<b>Lactate</b>
Blank	7.7(1.8-15.3)	2.5 (0.2-6.2)	0.7(0.0-2.4)	0 (0.0-0.0)
Glucose	49.1(23.7-120.5)	11.4 (0.0-25.0)	0.8 (0.0-3.5)	13.7 (0.0-73.1)
Lactose	54.5 (26.5-128.6)	7.5 (0.0-35.6)	0.3 (0.0-4.6)	21.4 (0.0-114.3)
Raftilose™	35.7 (15.5-106.3)	8.0 (0.5-34.2)	1.9 (0.0-24.5)	7.6 90.0-47.2)
Soyabean polysaccharide	16.8 (6.0-25.0)	5.7 (1.6-13.9)	0.7 (0.0-3.3)	0 (0-34)
Pectin	38.7 (7.3-68.3)	11.4 (2.3-20.5)	0.8 (0.0-6.0)	0 (0.0-74.1)
Starch	11.9 (5.1-50.1)	4.0 (0.5-12.6)	0.5 (0.0-8.8)	0 (0.0-27.7)
<b>Early weaning</b>	<b>Acetate</b>	<b>Propionate</b>	<b>n-Butyrate</b>	<b>Lactate</b>
Blank	7.4 (1.4-17.6)	2.3 (0.5-6.8)	1.1 (0.0-2.5)	0 (0.0-0.0)
Glucose	44.9 (25.9-75.8)	13.2 (1.4-33.1)	3.3 (0.0-20.7)	3.1 (0.0-55.9)
Lactose	52.8 (25.3-118.1)	14.2 (0.7-53.4)	2.0 (0.0-20.0)	0.0 (0.0-37.4)
Raftilose™	35.8 (11.7-60.0)	12.2 (1.2-28.8)	3.0 (0.0-13.8)	0.0 (0.0-49.6)
Soyabean polysaccharide	19.7 (9.6-28.1)	6.3 (1.5-11.6)	2.4 (0.0-6.8)	0.0 (0.0-4.0)
Pectin	39.2 (4.8-61.6)	9.7 (2.0-19.6)	1.7 (0.0-6.5)	0.0 (0.0-9.1)
Starch	26.6 (8.1-51.9)	5.7 (0.8-33.6)	2.7 (0.0-16.3)	0.0 (0.0-15.7)
<b>Late weaning</b>	<b>Acetate</b>	<b>Propionate</b>	<b>n-Butyrate</b>	<b>Lactate</b>
Blank	8.9 (2.3-91.9)	2.0 (0.3-5.7)	2.0 (0.0-4.1)	0.0 (0.0-0.0)
Glucose	42.9 (15.9-96.4)	5.4 (1.4-33.6)	9.3 (0.0-21.7)	1.9 (0.0-48.0)
Lactose	41.3 (26.0-108.4)	5.3 (1.1-38.6)	10.9 (0.0-21.8)	0.0 (0.0-46.0)
Raftilose™	42.1(9.3-106.4)	5.1 (2.0-28.2)	7.9(0.3-29.5)	0.0 (0.0-54.1)
Soyabean polysaccharide	22.3 (2.3-59.1)	6.7 (1.5-17.6)	3.4 (0.6-8.1)	0.0 (0.0-9.6)
Pectin	52.4 (25.4-110.1)	11.9 (4.8-20.5)	4.5 (0.3-13.7)	0 (0.0-10.1)
Starch	24.1 (9.8-79.5)	5.3 (1.6-14.5)	3.91 (1.3-133)	0 (0.0-15.7)
<b>Very late weaning</b>	<b>Acetate</b>	<b>Propionate</b>	<b>n-Butyrate</b>	<b>Lactate</b>
Blank	9.1 (4.8-13.2)	2.2 (1.4-4.3)	2.2 (0.8-4.0)	0 (0.0-0.0)
Glucose	38.9 (13.3-62.0)	4.4 (0.7-24.1)	5.9 (0.0-23.4)	5.0 (0.0-91.3)
Lactose	48.6 (36.2-69.0)	4.4 (0.7-28.6)	2.4 (0.0-1.0)	10.6 (0.0-89.3)
Raftilose™	40.9(30.7-60.4)	5.9 (2.8-26.1)	6.9 (1.0-37.8)	0.0 (0.0-34.3)
Soyabean polysaccharide	24.6 (10.5-41.3)	6.9 (3.9-14.0)	3.8 (0.2-11.5)	0.0 (0.0-50.1)
Pectin	49.9 (13.0-66.29)	8.1 (4.1-16.1)	5.2 (0.3-18.4)	0.0 (0.0-29.6)
Starch	28.0 (4.9-61.5)	6.8 (1.7-18.0)	6.9 (0.0-22.2)	0.0 (0.0-8.7)

### 7.3.3.5 Proportions of SCFA

In general the proportions of acetate for each substrate remained constant throughout (Table 7.22). Propionate proportions increased with the simple sugars and oligosaccharide between pre- and early weaning but decreased with complex

carbohydrates. Proportions of propionate then decreased at late and early weaning with a simultaneous increase in butyrate. Lactate was not produced with complex carbohydrates and is produced mainly at pre-weaning with the simple sugars and oligosaccharide.

**Table 7.22** Proportions of individual SCFA produced in *in vitro* cultures of faecal bacteria in mixed fed infants (n=19)

Pre-weaning	Acetate	Propionate	n-Butyrate	Lactate
Blank	55.4 (24.4-78.8)	19.1 (4.0-48.5)	6.5 (0.0-16.0)	0.0 (0.0-0.2)
Glucose	58.8 (36.6-79.9)	18.3 (0.0-41.2)	0.9 (0.0-7.7)	20.6 (0.0-63.4)
Lactose	66.1 (24.6-86.2)	10.0 (0.0-42.5)	0.4 (0.0-6.2)	23.6 (0.0-71.0)
Raftilose™	58.5 (36.2-87.4)	11.4 (0.7-38.0)	2.7 (0.0-44.8)	10.7 (0.0-50.6)
Soy.polysacc.	62.9 (39.2-84.3)	24.9 (6.3-43.7)	3.1 (0.0-14.4)	0.0 (0.0-9.7)
Pectin	71.2 (28.7-92.8)	21.3 (4.4-35.7)	1.6 (0.0-6.9)	0.0 (0.0-63.4)
Starch	67.6 (35.0-85.6)	16.6 (4.9-45.4)	2.6 (0.0-13.0)	0.0 (0.0-50.0)
Early weaning	Acetate	Propionate	n-Butyrate	Lactate
Blank	56.2 (18.4-76.9)	16.4 (4.3-34.2)	8.4 (0.0-18.9)	0.0 (0.0-0.0)
Glucose	62.9 (44.4-94.9)	17.4 (3.9-30.1)	4.4 (0.0-28.9)**	3.8 (0.0-34.4)
Lactose	67.2 (54.3-97.3)	18.3 (2.7-30.4)	3.0 (0.0-33.9)*	0.0 (0.0-23.9)*
Raftilose™	64.7 (42.7-88.7)	20.6 (2.3-34.9)	4.7 (0.0-36.1)	0.0 (0.0-36.1)
Soy.polysacc.	60.7 (34.0-78.3)	20.4 (9.5-34.0)	8.0 (0.0-18.2)*	0.0 (0.0-12.3)
Pectin	73.1 (26.0-84.5)	19.2 (5.9-47.7)	3.5 (0.0-13.9)	0.0 (0.0-24.9)
Starch	64.2 (44.6-85.6)	17.6 (4.3-34.9)	8.7 (0.0-21.8)	0.0 (0.0-20.6)
Late weaning	Acetate	Propionate	n-Butyrate	Lactate
Blank	59.3 (49.7-94.9)	13.5 (1.4-24.6)*	12.9 (0.0-19.4)**†	0.0 (0.0-0.0)
Glucose	62.4 (24.1-81.4)	10.8 (2.1-32.3)	11.4 (0.0-32.2)**	2.7 (0.0-73.0)
Lactose	64.1 (42.7-97.3)	9.8 (2.6-27.3)†	10.9 (0.0-28.5)**	0.0 (0.0-53.2)
Raftilose™	65.8 (14.1-90.6)	11.6 (3.3-36.1)†	11.3 (0.5-25.0)	0.0 (0.0-82.2)
Soy.polysacc.	63.3 (13.1-74.6)	16.9 (7.7-57.7)	9.5 (3.4-23.8)**	0.0 (0.0-20.8)
Pectin	74.7 (56.7-89.5)	14.7 (6.8-30.9)††	6.6 (0.8-17.3)**†	0.0 (0.0-12.0)
Starch	61.4 (50.9-76.3)	15.7 (9.1-25.5)	11.2 (2.7-27.9)***	0.0 (0.0-33.3)
Very late weaning	Acetate	Propionate	n-Butyrate	Lactate
Blank	59.1 (33.0-69.5)	15.0 (10.1-19.9)	11.7 (6.5-19.0)**†	0.0 (0.0-0.0)
Glucose	59.8 (39.1-88.1)	7.7 (1.0-32.3)†	7.0 (0.0-26.5)***	10.4 (0.0-57.52)
Lactose	65.7 (41.3-87.5)	5.9 (0.8-30.0)††	2.2 (0.0-20.2)**	14.8 (0.0-56.5)
Raftilose™	64.9 (44.1-83.3)	10.6 (4.2-34.3)†	11.8 (2.1-44.9)*	0.0 (0.0-35.6)
Soy.polysacc.	60.2 (28.6-69.8)	22.0 (9.0-28.5)*	10.7 (0.9-23.6)**†	0.0 (0.0-58.3)
Pectin	73.8 (48.4-85.9)	14.0 (8.2-24.1)**††	10.0 (1.4-19.1)**†	0.0 (0.0-32.2)
Starch	60.7 (52.5-68.6)	18.2 (9.3-25.6)	12.5 (0.0-30.1)**†	0.0 (0.0-15.6)

\*p<0.05, \*\*p<0.01 compared with pre-weaning; †p<0.05 compared with early weaning; ‡ p<0.05 compared with late weaning. Soy. Polysacc.= soyabean polysaccharide

### 7.3.3.6 Differences between substrates

Most substrates showed significant differences between one another when total SCFA and lactate amounts were compared at pre and early weaning (Table 7.23). Most of these significant differences continued through late and very late weaning.

**Table 7.23 Differences in total SCFA production including lactate between substrates**

<b>Pre-weaning</b>	Glucose	Lactose	TM	Soyabean polysaccharide	Pectin	Starch
Glucose		↓*	↑**	↑***	↑**	↑***
Lactose	↑*		↑***	↑***	↑**	↑***
Raftilose <sup>TM</sup>	↓**	↓***		↑***	↑	↑***
Soyabean polysaccharide	↓***	↓***	↓***		↓***	↑*
Pectin	↓**	↓***	↓	↑***		↑**
Starch	↓***	↓***	↓***	↓	↓**	
<b>Early weaning</b>						
Glucose		↑	↑**	↑***	↑*	↑**
Lactose	↓		↑**	↑***	↑*	↑**
Raftilose <sup>TM</sup>	↓**	↓**		↑***	↑	↑*
Soyabean polysaccharide	↓***	↓***	↓***		↓*	↓
Pectin	↓*	↓*	↓	↑*		↑
Starch	↓**	↓**	↓*	↑	↓	
<b>Late weaning</b>						
Glucose		↓*	↑	↑**	↓	↑***
Lactose	↑*		↑	↑***	↑	↑***
Raftilose <sup>TM</sup>	↓	↓		↑**	↓	↑**
Soyabean polysaccharide	↓**	↓***	↓**		↓***	↓
Pectin	↑	↓	↑	↑***		↑***
Starch	↓***	↓***	↓**	↑	↓***	
<b>Very late weaning</b>						
Glucose		↓	↑	↑**	↓	↑**
Lactose	↑		↑*	↑***	↑*	↑***
Raftilose <sup>TM</sup>	↓	↓*		↑**	↓	↑**
Soyabean polysaccharide	↓**	↓***	↓**		↓**	↓
Pectin	↑	↓*	↑	↑**		↑**
Starch	↓**	↓***	↓**	↑	↓**	

### 7.3.5 Differences between breast-fed, formula-fed and mixed fed infants

**Table 7.24** Comparisons of infant feeding groups for total SCFA including lactate

	Median values $\mu\text{moles/ml}$			
	Pre-weaning	Early Weaning	Late Weaning	Very Late Weaning
<b>Breast-fed</b>				
Glucose	66.3	63.0	59.0	52.7
Lactose	76.5	75.3	71.2	61.1
Raftilose™	45.2	48.7	59.8	56.1
Soy	10.0	12.9	19.9	25.7
polysaccharide				
Pectin	18.6	32.3	50.9	53.1
Starch	7.3	8.1	16.4	27.3
<b>Formula-fed</b>				
Glucose	75.4	52.1	62.4	52.7
Lactose	83.1	54.8	64.5	65.5
Raftilose™	69.53	44.5	59.8	55.9
Soy	15.0	20.2	20.1	23.5
polysaccharide				
Pectin	41.9	48.0	46.3	50.2
Starch	7.1	12.0	21.3	25.4
<b>Mixed fed</b>				
Glucose	67.8	55.3	59.5	55.4
Lactose	76.7	54.8	63.1	57.6
Raftilose™	50.8	48.7	50.7	51.3
Soy	11.8	17.3	18.3	25.2
polysaccharide				
Pectin	47.9	40.1	62.8	55.1
Starch	6.0	27.1*	21.1	26.7

\*p < 0.05 compared with breast-fed infants

#### **7.3.4.1 pH**

No differences were seen between the feeding groups at any age for decrease in pH from blank.

#### **7.3.4.2 Total SCFA including lactate**

The only significant difference (Table 7.24) between the three infant feeding groups when comparing total SCFA including lactate was with cultures of starch in early weaning, breast-fed had significantly less than mixed fed infants ( $p < 0.05$ ).

#### **7.3.4.3 Total SCFA without lactate**

There were no changes in significant differences between total SCFA including lactate and without lactate.

#### **7.3.4.4 Individual SCFA**

Formula-fed and mixed fed infants produced more propionate and butyrate at pre- and early weaning than breast-fed infants. By late and very late weaning the concentrations of each SCFA for all feeding groups were similar.

#### **7.3.4.5 Proportions of individual SCFA**

At pre and early weaning, proportions of butyrate were higher in formula-fed and mixed fed infants than breast-fed infants. At pre-weaning this was also true of propionate. By late weaning breast-fed infants were producing similar proportions of all SCFA as formula-fed infants. At early and late weaning mixed fed infants were producing higher proportions of butyrate. It is only at very late weaning that proportions of all SCFA for all feeding groups are similar.



#### **7.3.4.6 Differences between substrates**

Similar differences were seen between substrates in the three feeding groups. However there were less differences with faecal cultures of formula-fed infants than with breast-fed or mixed fed infants particularly at pre- and early weaning.

### **7.4 DISCUSSION**

Faecal SCFA represent the net sum of digestion, production and absorption of SCFA throughout the gastrointestinal tract. In infants the microflora is not stable and changes in response to new foods are introduced. Once the bacterial flora of the adult is established it tends to remain stable and does not change significantly with diet (Bornside, 1978). Weaning is likely to be a critical period in the development of the adult flora.

There is already some information about the flora of breast-fed and formula-fed infants in the first weeks of life (Edwards *et al.*, 1994; Balmer and Wharton, 1989; Schmitz and McNeish, 1987) but little is known about how the flora changes at weaning. Less is known about mixed fed infants either in the first weeks of life or during the weaning process.

In infancy a significant amount of carbohydrate, mainly lactose escapes digestion in the small intestine and becomes available for fermentation in the colon. The colonic bacteria may also ferment endogenous mucus and mucopolysaccharides as well as fructo-oligosaccharides in breast milk and substantial amounts of maltodextrins some infant formula. The response of the flora of the infant at weaning may depend on previous feeding practice. It is important to know how changes in microflora allow infants to cope with new substrates they meet at weaning. New carbohydrates in the diet may have an influence the composition and metabolic activity of the microflora.

As breast-fed and formula-fed infants differ in terms of bacterial populations and fermentation patterns it might be expected that this would affect their ability to ferment new carbohydrates. If fermentation capacity is sufficiently developed the SCFA will provide 8.4 kJ (2 kcal)/g contributing to daily energy needs (Livesey, 1990). If infants are presented with a substrate they are unable to ferment, it will pass through unmetabolised pulling water with it. This will cause an increase in stool output which may result in increased faecal energy loss. Although breast-fed infants are less prone to diarrhoea at weaning than formula-fed infants, at weaning it is breast-fed infants who may be more susceptible to gastrointestinal infection (Gordon, 1971). Weaning may have less effect on formula-fed infants as their microflora is closer to that of adults even before weaning.

In this longitudinal study we investigated the ability of three different infant feeding groups, breast-fed, formula-fed and mixed fed to ferment simple and complex carbohydrates through the weaning process. The differences between the three groups of infants were also studied at each of the development stages.

Although fermentation capacity had been investigated previously (Chapter 2) it had only been in a small group of infants in a study of cross-sectional design. It was concluded from that study that a larger sample size and a longitudinal design were needed to verify results. The study described in this chapter included breast-fed, formula-fed and mixed fed infants. Mixed fed infants have not been studied in any great detail with regard to bacterial colonisation and fermentation capacity previously. With the continued emphasis on the benefits of breast-feeding there are consequently a large number of infants who receive both human and formula milk before weaning. This group of infants is very important in the UK, as more mothers begin to breast-feed but are then, for a variety of reasons, unable or unwilling to continue. It was unclear whether this change from breast to formula milk would cause a change in the colonic flora and a subsequent change in fermentation capacity.

By following infants from birth we were able to obtain samples for *in vitro* fermentation at a number of development stages. Following the same infants at these stages would enable a clearer idea of the changes that were occurring as weaning progressed. In addition to glucose, raftilose™ and soyabean polysaccharide substrates that had been investigated in the pilot study, lactose, pectin and starch were also used as substrates.

However the design of the study described here meant that there were three feeding groups, four development stages and seven substrates to be investigated. This created numerous results and after consultation with statisticians it was agreed that statistical tests could only be used on some of the data. By using statistical tests on each combination of the data there was a possibility of significant differences occurring by chance. Statistical tests have been carried out on a large amount of the data to gain meaningful results, because of this more emphasis should be put on significant differences of  $p < 0.01$  than  $p < 0.05$ .

In all groups of infants, the decreases in pH were similar. Even at pre-weaning there was a considerable decrease in pH for the simple sugars and raftilose™. In contrast, with the soyabean polysaccharide and starch, the final pH was similar to that of the control culture even at very late weaning. Pectin at pre-weaning had a similar pH to the control culture but by early weaning a decrease in pH was observed. This suggests that in all infant feeding groups the simple sugars and raftilose™ are fermented equally well from pre-weaning, the fermentation capacity for pectin appears to increase in early weaning, and even by very late weaning the fermentation capacity for soyabean polysaccharide and starch is still poor.

Total SCFA including lactate concentration in all infant groups with cultures of the simple sugars or raftilose™ was high even at pre-weaning. This suggests that infants of all feeding groups are equally able to ferment glucose, lactose and raftilose™ from

pre-weaning. This is in contrast to the pilot study where there is a bigger increase in *raffilose*<sup>TM</sup> fermentation at early weaning in breast-fed infants (Chapter 2).

The ability of formula-fed and mixed fed infants to ferment pectin was from pre-weaning, in contrast the breast-fed infants appeared to not be able to ferment pectin until early weaning. For cultures with starch and soyabean polysaccharide fermentation capacity did not increase until early weaning with formula-fed and mixed fed infants and did not increase until late weaning with breast-fed infants. Even when lactate concentration was not included in total SCFA these findings were the same.

When proportions of individual SCFA were investigated, it was seen that there were differences between the three feeding groups. The breast-fed and mixed fed group produced mainly acetate and lactate with the simple sugars and *raffilose*<sup>TM</sup> at pre-weaning whereas the formula-fed had high acetate, propionate and lactate. With the complex carbohydrates mainly acetate and propionate was produced by the breast-fed infants; acetate, propionate and a small amount of butyrate was produced by the formula-fed and mixed fed infants.

For simple sugars and *raffilose*<sup>TM</sup> at early weaning in breast and mixed-fed infants acetate was still produced but propionate increased at the expense of lactate. In formula-fed infants acetate and propionate were the dominant SCFA as at pre-weaning although there was some increase in butyrate and lactate was still present. With the complex carbohydrates all three groups were producing acetate, propionate and small amounts of butyrate.

By late and very late weaning for all substrates and all feeding groups acetate was the main SCFA but propionate and butyrate also produced. By these stages very little propionate is seen.

Most statistical differences occurred in the proportions of propionate and butyrate. Butyrate increased markedly in all three feeding groups with all substrates over the weaning period.

## **7.5 CONCLUSIONS**

The study described in this chapter shows that the simple sugars and raftilose™ are equally well fermented by all three infant groups at each development stage. Fermentation of glucose by infants confirmed results of the cross-sectional pilot study (Chapter 2). However, breast-fed infants appeared to be able to ferment raftilose™ better at pre-weaning than was originally shown in the pilot study, although there was a significant increase in total SCFA produced between early and late weaning.

The ability to ferment complex carbohydrates differed between the three infant feeding groups. With cultures containing soyabean polysaccharide, similar results were shown to those of the cross-sectional pilot study (Chapter 2). Soyabean polysaccharide was less well fermented throughout weaning. In breast-fed infants there was a significant increase in total SCFA produced with soyabean polysaccharide at late weaning whereas in mixed fed infants the ability to ferment it increased at early weaning. In contrast there were no differences seen in the formula-fed infants throughout weaning. By very late weaning the total SCFA produced was similar in all infant feeding groups but was very low (approximately 50% of values from cultures containing glucose). Infants were still not achieving levels previously seen in adults (Chapter 2). The ability of infants to ferment starch was followed a similar pattern. In breast-fed and mixed fed infants the ability to ferment pectin increased significantly at late weaning, however, the total SCFA produced by mixed fed infants at pre-weaning was much greater than breast-fed infants although this did not reach significance. Formula-fed infants had no differences in total SCFA production throughout weaning with cultures containing pectin.

Formula-fed appear to adapt to the complex carbohydrates quickest, followed by mixed fed infants with the breast-fed infants adapting more slowly. The greater ability of formula-fed infants to ferment complex carbohydrate at pre-weaning is probably a reflection of the greater similarity of their flora to that of the adult. This would suggest therefore, that mixed fed infants by early weaning were developing a flora more like that of an adult. The slow adaptation of the breast-fed infant group may be correlated with the higher incidence of diarrhoea in breast-fed infants at weaning (Gordon, 1971).

Due to breast-feeding promotion more mothers are beginning to initially feed their infants with human milk. However, some are unable or unwilling to continue and consequently infants being fed a mixture of breast and formula is increasing. Very few investigations have been carried out in mixed fed infants previously so knowledge of the colonic and faecal flora in this group of infants is limited. Although their fermentation ability is similar to that of formula-fed infants the profile of SCFA is not the same, therefore they should be considered as a distinct group.

In conclusion the microbial colonisation of the lower digestive tract of infants is a complex process. The fermentation of the complex carbohydrates soyabean polysaccharide and starch was poor even at very late weaning. In contrast the ability to ferment pectin, another complex carbohydrate was greater in all infant feeding groups. The development of the ability to ferment carbohydrates therefore depends both on the complexity of the carbohydrate and the bacteria dominating the microflora. As weaning progresses the colonic microflora changes and the ability to ferment carbohydrates develops. The ability to ferment complex carbohydrates in infants suggests that the maturation of the colonic flora and its fermentation capacity is a slow process. This is supported by studies indicating bacterial activities such as degradation of mucus, conversion of bilirubin to urobilinogen, metabolism of cholesterol to coprostanol and inactivation of faecal tryptic activity were still increasing at two years of age in Swedish children (Midtvedt et al., 1988, 1994;

Midtvedt and Midtvedt, 1992). The slow development of fermentation capacity suggests that the colonic salvage of energy from complex carbohydrates is unlikely to contribute significantly to daily energy intake of infants. Sugars and oligosaccharides however which are well fermented, if undigested, will contribute energy in the form of SCFA. The fermentation model used in this study allowed fermentation over 24 hours. In some individuals transit time may exceed this so carbohydrates would be exposed to the colonic flora for longer which may allow greater fermentation.

Continued ingestion of complex carbohydrates may alter the colonic flora of infants, inducing enzymes and altering bacterial populations which may increase the ability to ferment these substrates. If weaned onto a high-fibre diet the infants may have developed a flora better able to ferment complex carbohydrates. In the first year of life when the colonic microflora is developing it could be manipulated by diet to give future health benefits.

## **Chapter 8**

### **Discussion and conclusions**



## 8.1 INTRODUCTION

Carbohydrates that escape digestion and absorption in the small intestine pass through to the colon where they may be fermented by colonic flora. This process produces SCFA (Edwards *et al.*, 1994), the absorption of which salvages energy and prevent osmotic water loss (Kien *et al.*, 1990). Carbohydrate fermentation in adults is thought to have an energy value of 2 Kcal/ g (Livesey, 1990). Bacterial colonic fermentation in adults has been well investigated but knowledge of the colonic flora and how it develops in infants and during weaning is limited.

It is thought that birth and weaning are critical stages in the development of the gut microflora. The colonisation of the intestine in the first year of life may also determine the properties of the final adult microflora, which could have major health implications. Thus it is essential that we have a good understanding of the development of bacterial colonisation of the infant gut and the maturation of the metabolic capabilities of the resultant flora.

As discussed in Chapter 1, the current published knowledge is limited, in particular, in respect to the development of fermentation capacity in infants.

The main objectives of this thesis therefore were:

1. To carry out a cross-sectional pilot study of the development of fermentation capacity in breast-fed and formula-fed infants.
2. Using the data from the pilot study to improve design, to carry out a longitudinal study of the development of fermentation capacity in three infant feeding groups (breast-fed, formula-fed and mixed fed) for a range of simple and complex carbohydrates.
3. To measure faecal SCFA in different groups of infants (breast-fed, formula-fed and mixed fed) followed from birth to 18 months of age.

4. To measure faecal starch in different groups of infants (breast-fed, formula-fed and mixed fed) followed from birth to 18 months of age.
5. To measure faecal fat in breast-fed and formula-fed infants in the first year of life.

*In vivo* methods are not suitable for study of infant samples because of the invasive nature of the techniques. Therefore in order to measure fermentation capacity it was necessary to use an *in vitro* model.

## **8.2 IN VITRO MODEL**

Several possible models were considered as discussed in Chapter 3. A modified model from that of Adiotomre *et al.*, 1990 was chosen to determine the ability of infants to ferment a range of simple and complex carbohydrates because it was relatively simple, rapid and cheap. This allowed a large number of samples to be fermented at once. In addition, it was a method that enabled the use of smaller faecal samples which were likely to be obtained from infants, especially breast-fed infants. The media contained all the potential growth requirements of this simplified flora which had not been included in the methods of Barry *et al.*, and Edwards *et al.* (Chapter 3). In those models designed for adult studies, the faeces was assumed to provide all micronutrients, but this may not be the case in the much smaller infant faecal samples.

Obviously using an *in vitro* model has some limitations. It is very limited in its ability to mimic the events in the lumen of the colon. Although models which allowed shaking and mixing of the culture to mimic intestinal motility were considered it was not practical to use them because of the large number of samples involved. Therefore the model chosen was static, and although lack of mixing will slow the rate of fermentation, an incubation of 24 hours is long enough to overcome. As there was no metabolite absorption there was a possibility of product inhibition of fermentation by accumulated SCFA and low pH. This model also had a limited

carbon supply. However there may also have been a change in bacterial dominance as the culture aged.

Taking into account the advantages and disadvantages, this model chosen was the best one to use with the infant samples.

### **8.3 CROSS-SECTIONAL PILOT STUDY**

The main experiment in this thesis was designed on the basis of results of a cross-sectional pilot study. The results of the pilot study indicated that there were differences in the fermentation capacity between breast-fed and formula-fed infants. Although both groups of infants could ferment glucose equally well, in the breast-fed infants the capacity to ferment *raffinose*<sup>TM</sup>, a fructo-oligosaccharide, did not increase until early weaning ( $p < 0.05$ ) and the capacity to ferment soyabean polysaccharide did not increase until late weaning ( $p < 0.001$ ). Both groups of infants produced less total SCFA with complex carbohydrate than adults. This suggested that the fermentation capacity in infants was still developing at late weaning. However this study was of cross-sectional design and had small numbers ( $n=10$ ) and only breast-fed and formula-fed infants were investigated up to the age of one year. It was decided to verify the results, a larger group of infants should be investigated for a longer period and the study design should be longitudinal. As there are a large number of infants in the UK that receive both human and formula milk, it was important to study the development of fermentation capacity in these infants too. In addition a larger range of simple and complex carbohydrates were investigated in the longitudinal study to give more information about the relative fermentation capacities of carbohydrates.

## **8.4 LONGITUDINAL STUDY**

### **8.4.1 Characteristics of sample**

To consider the influence of age and diet on the flora it would be ideal if the infants studied were equal in all other respects. By comparing various characteristics of the infants and mothers the similarity of the infants in each feeding group was investigated.

The three infant feeding groups, breast-fed, formula-fed and mixed fed, were shown to be comparable in all characteristics apart from a minor difference in birth weight (breast-fed lower than mixed fed infants,  $p < 0.05$ ). As the characteristics of the feeding groups were similar, comparisons of faecal SCFA and fermentation capacities could be made on the basis of feeding practice alone.

The mothers who took part in the study were of higher social class, older and more likely to breast-feed ( $p < 0.01$ , 0.01, 0.05 respectively) than the non-participants but this must be expected in a study where subject motivation and co-operation were necessary. Although not a perfect match, the sample was a reasonable representation of the Glasgow area when neighbour-hood type 1+2, 3+4, 5+6, 7+8 were considered together.

### **8.4.2 Development of fermentation capacity in infants**

#### **8.4.2.1 Fermentation capacity**

Total SCFA including lactate concentration was high even at pre-weaning in cultures containing simple sugars for all infant feeding groups. The amount of SCFA including lactate produced from raftilose™ increased between early and late weaning

for breast-fed infants ( $p < 0.05$ ), whereas there were no significant differences in formula-fed and mixed fed infant groups. Pectin fermentation increased between pre- and late ( $p < 0.01$ ) and early and late ( $p < 0.05$ ) for breast-fed infants. In mixed fed infants there was a significant increase between early and late weaning and between early and very late weaning (both  $p < 0.01$ ). For formula-fed infants there were no significant differences between any development stages. For the other carbohydrates, soyabean polysaccharide and starch, SCFA were produced at low concentrations throughout weaning, although increases were seen between the development stages. These differences were significant for breast-fed infants at late weaning when compared to pre- ( $p < 0.05$  for soyabean polysaccharide,  $p < 0.01$  for starch) and early weaning ( $p < 0.05$ ). SCFA also increased between very late weaning and pre- ( $p < 0.01$  for soyabean polysaccharide,  $p < 0.05$  for starch), early ( $p < 0.001$ ,  $0.01$  respectively) and late weaning ( $p < 0.05$  for soyabean polysaccharide). For mixed fed infants increases were significant between early and late weaning compared ( $p < 0.05$ ) with pre-weaning in cultures of soyabean polysaccharide and between early and pre-weaning with starch ( $p < 0.01$ ). The only significant difference in formula-fed infants was between late and pre-weaning with starch ( $p < 0.05$ ).

This confirms the findings of the cross-sectional study in respect of glucose and soyabean polysaccharide. Although total SCFA increased between early and late weaning with cultures containing raftilose™ the increased ability to ferment this substrate at early weaning seen in the pilot study was not apparent.

#### **8.4.2.2 Patterns of fermentation**

When proportions of individual SCFA were compared the percentage of propionate and butyrate increased for most substrates from pre-weaning and early weaning in faecal cultures of breast-fed infants. The general trend was for propionate and butyrate to increase significantly between pre- and early weaning with the simple

sugars and oligosaccharide and between pre and late weaning for the complex carbohydrates.

Few changes were seen in proportions of propionate with formula-fed infants although butyrate increased significantly from pre-weaning to late weaning. In mixed fed infants, propionate proportions increased in late and very late weaning from early weaning with cultures of lactose, raftilose™ and pectin. Increases in the proportion of butyrate were seen between early, late and very late weaning from pre-weaning values with most substrates. Considering the importance of butyrate in the adult colon it is interesting that the proportion of butyrate is very low especially at pre- and early weaning in breast-fed infants and at pre-weaning in mixed fed infants. This suggests that butyrate may not be as important for the infant colon as it is in the adult.

Lactate was produced by all infant feeding groups in cultures containing glucose and lactose before weaning. Although for breast-fed and formula-fed infants lactate remained in cultures with these substrates throughout weaning it was not present in faecal cultures of mixed fed infants from early weaning onwards. Cultures containing raftilose™ also produced lactate in formula-fed infants throughout weaning and with mixed fed infants at pre weaning only. Lactate proportions before weaning were much higher in breast-fed infants.

When compared with adult concentrations (Chapter 2) glucose and raftilose™ produced similar concentrations of SCFA in all groups of infants. In contrast, soyabean polysaccharide produced much lower concentrations of SCFA than adults in all infant feeding groups.

#### **8.4.3 Age vs weaning**

It is possible that the development of bacterial activity increases as a direct result of increasing age rather than as an effect of weaning. It is, however, difficult to separate

the effects of age from the effects of weaning. To be able to do this a large number of infants who had been weaned at different ages or who had had weaning delayed would be needed. In this study most mothers weaned at about the same age (3-4 months) and although there were a few mothers who weaned much later (> 5 months) they were from all three feeding groups. It would be appropriate to comment on the results of individual infants as they may not be representative.

#### **8.4.4 Faecal SCFA in infants**

The total faecal SCFA including lactate significantly increased between pre- and all other development stages in breast-fed infants ( $p < 0.01$ ) and in mixed fed infants ( $p < 0.05$ ). In contrast, no differences were seen in formula-fed infants between any development stage.

In breast-fed infants, the proportions of both propionate and butyrate significantly increased through the weaning stages ( $p < 0.01$ ) and lactate significantly decreased ( $p < 0.001$ ). There was also a significant decrease in proportion of acetate between early and very late weaning ( $p < 0.05$ ). Fewer changes were seen in formula-fed infants although there was a significant increase in butyrate at the later weaning stages from the early weaning stages ( $p < 0.05$ ). Similarly in mixed fed infants there was a significant increase in butyrate throughout the weaning stages ( $p < 0.01$ ). As with breast-fed infants, there was a significant decrease in acetate from pre- to very late weaning ( $p < 0.01$ ).

When total faecal SCFA were compared there were no differences in the three infant feeding groups between any development stage. However, breast-fed infants had significantly lower proportions of propionate ( $p < 0.001$  at pre-weaning and  $p < 0.01$  at early weaning) and butyrate ( $p < 0.001$ ,  $p < 0.01$  for formula-fed infants at pre- and early weaning;  $p < 0.05$  for mixed fed infants at pre- and early weaning) than formula or mixed fed infants. Mixed fed infants also had significantly lower proportions of

butyrate ( $p < 0.05$ ) at pre-weaning than formula-fed infants. In addition breast-fed infants had significantly higher proportions of lactate at pre- ( $p < 0.001$ ) and early weaning ( $p < 0.01$ ) than formula-fed infants and at pre-weaning ( $p < 0.001$ ) than mixed fed infants.

This study confirms previous findings that the faecal SCFA of breast-fed and formula-fed infants is different prior to weaning (Edwards *et al.*, 1994). The breast-fed infants had a profile dominated by acetate and lactate whereas formula-fed infants have acetate, propionate and butyrate present. Mixed fed infants had a similar profile to formula-fed infants but with more propionate and less butyrate. At early weaning, breast-fed and formula-fed faecal SCFA differences remained whereas mixed fed infants had become more similar to the formula-fed infant. By late weaning proportions of acetate, propionate and butyrate are similar in all three feeding groups and no lactate was present.

This suggests that the mixed fed infant group is different to both the breast-fed and formula-fed infants and should be considered as a distinct group. As many infants are mixed fed rather than exclusively breast-fed, more investigations need to be undertaken in this group to study the both the profile and activity of their colonic microflora.

#### **8.4.5 Faecal starch excretion**

Faecal starch is starch that has escaped both digestion and fermentation. Excretion of starch in faeces has been seen previously in infants up to 3 years of age (Verity and Edwards, 1994). Very little starch is excreted before weaning as infants are not exposed to starch in the diet. In the study in this thesis, at early weaning when the weaning process was still in its early stages there was an increase in faecal starch excreted by all infant feeding groups. This was higher in breast-fed and mixed fed infants than in formula-fed infants (although this difference reaches significance only



with mixed fed infants). This suggests that the formula-fed infants have a better ability to cope with starch that is introduced into the diet than the other two feeding groups. Again at late weaning there is a significant increase in faecal starch from early weaning but there are no differences between any of the feeding groups. By very late weaning the amount of excreted starch is decreasing from late weaning in all feeding groups, although it is still significantly higher than at pre-weaning. This decrease is probably due to the maturation of the gut and less starch is therefore escaping digestion and passing through to the colon. In addition the colonic flora may be more able to ferment any starch entering the colon. The decrease was most marked in the formula-fed and mixed fed infants although it was not significant, suggesting that the digestive capacities of the breast-fed infant was still maturing. This starch which has escaped both digestion and fermentation may be increase stool output and increase faecal energy losses.

#### **8.4.6 Faecal fat excretion**

Formula-fed infants had significantly lower faecal fat excretion at late weaning than pre-weaning, whereas no differences were seen in breast-fed infants through the development stages. Formula-fed infants excreted more fat in faeces than breast-fed infants at both pre- and early weaning but by late weaning no differences were seen.

More faecal fat was excreted by formula-fed infants than breast-fed infants at pre and early weaning, by late weaning both groups were excreting very small amounts. This was probably due to the increased ability of breast-fed infants to digest fat because of the type of fat in breast milk and the presence of a bile-salt stimulated lipase in breast-milk.

The high digestibility of human milk has been shown to be related to the presence of bile salt-stimulated lipase in human milk (Hernell and Blackberg, 1982), the structure of the milk fat globule (Gaull *et al.*, 1982) and to the sn-2 configuration of palmitic

acid in the triglyceride (Filer *et al.*, 1969). Formula milk fats are composed of vegetable fat which have sn-1,3 position in the triglyceride. The bile salt-stimulated lipase and the structure of the milk fat globule improve triglyceride lipolysis in breast-fed infants. The sn-2 configuration of palmitic acid allows the absorption of palmitate as the 2-monoglyceride from human milk rather than the free acid.

Differences in lipids between formula- and breast-fed infant stools has been shown to be almost entirely due to fatty acids, mainly C16:0 and C18:0 (Quinlan *et al.*, 1995). The authors suggested that this was due to the infants handling of saturated fatty acids.

Starch and fat lost in the faeces may represent a significant amount of energy loss for the infant. Although there is very little data on the stool output in early life and our data only represents a 'snapshot', it may be useful to roughly quantify these faecal energy losses. 1 g of starch and 1g fat releases 4 kcal and 9 kcal of energy respectively. From this it is possible to calculate the amount energy lost through starch and fat excretion. According to Weaver *et al.*, (1988b) infants, by 16 weeks of age, pass an average of two stools per day of mean size 10mls. If each ml of stool is converted to grammes this is equivalent of 20g of stools passed by infants each day. As the infant matures this stool will increase, so by 1 and 2 years (late and very late weaning respectively) they may be passing larger volumes of stool. As there is no available data for older children for the sake of this thesis, I will assume quantities of 100g per day. Estimated average energy requirements for energy (DOH, 1999) for males 4-6 months is 690 kcal and for girls 645 kcal. An average of these two figures, 668 kcal, is taken for energy intake for early weaning. Similar averages for energy intakes are taken for late weaning, 893 kcal, and very late weaning, 1198 kcal. Estimated faecal energy lost is shown in Tables 8.1, 8.2, 8.3.

**Table 8.1 Percentage of daily energy lost through faecal starch excretion**

	Early weaning			Late weaning			Very late weaning		
	Median	Min	Max	Median	Min	Max	Median	Min	Max
<b>Breast-fed</b>									
Faecal starch g/100g	0.6	0.02	1.49	1.38	0.13	2.75	1.04	0.12	5.37
Energy lost kcal	0.48	0.02	1.19	5.52	0.52	11.00	4.16	0.48	21.48
% energy lost	0.07	0.00	0.18	0.62	0.06	1.23	0.35	0.04	1.79
<b>Formula-fed</b>									
Faecal starch g/100g	0.2	0.02	2.33	1.12	0.32	2.92	0.57	0.14	2.32
Energy lost kcal	0.16	0.02	1.86	4.48	1.28	11.68	2.28	0.56	9.28
% energy lost	0.02	0.00	0.28	0.50	0.11	1.31	0.19	0.05	0.77
<b>Mixed fed</b>									
Faecal starch g/100g	0.74	0.26	3.43	1.17	0.55	3.28	0.50	0.00	2.10
Energy lost kcal	0.59	0.21	2.74	4.68	2.20	13.12	2.00	0.00	8.40
% energy lost	0.09	0.03	0.41	0.52	0.25	0.11	0.17	0.00	0.70

Values are given for faecal starch excretion g/ 100g faecal wet weight. This is converted to energy lost by 1g starch is equivalent to 4 kcal of energy and then divided by 5 to give an energy lost per 20g daily stool output for early weaning or left as per 100g for late and very late weaning. This is then converted to percentage of energy lost of estimated average requirements.

**Table 8.2 Percentage of daily energy lost through faecal fat excretion**

	Early weaning			Late weaning		
	Median	Min	Max	Median	Min	Max
<b>Breast-fed</b>						
Faecal fat g/100g	0.06	0.04	0.25	0.1	0.03	0.34
Energy lost	0.11	0.07	0.45	0.9	0.27	3.06
% energy lost	0.02	0.01	0.07	0.10	0.03	0.34
<b>Formula-fed</b>						
Faecal fat g/100g	0.30	0.09	0.52	0.12	0.03	0.29
Energy lost	0.54	0.16	0.94	1.08	0.27	2.61
% energy lost	0.08	0.02	0.14	0.12	0.03	0.29

Values are given for faecal fat excretion g/ 100g faecal wet weight. This is converted to energy lost by 1g starch is equivalent to 9 kcal of energy and then divided by 5 to give an energy lost per 20g daily stool output for early weaning or left as per 100g for late weaning. This is then converted to percentage of energy lost of estimated average requirements.

**Table 8.3 Estimation of percentage energy lost daily through faeces**

	Early weaning			Late weaning		
	Median	Min	Max	Median	Min	Max
Breast-fed	0.09	0.01	0.25	0.72	0.09	1.57
Formula-fed	0.10	0.02	0.42	1.22	0.20	1.60

Figures are an addition of faecal starch and fat excretion.

The maximum amount of energy lost for any infant would be 1.79 % of daily energy needs (calculated from the maximum starch excreted by one infant in the breast-fed group at early weaning). Although these are both estimates and a 'snapshot' of faecal losses it can be seen that the approximate energy lost through faecal losses from fat and starch excretion is minimal and would therefore not represent a problem to the health of the infants.

It was difficult from the limited dietary data collected during this study to relate food intake with starch and fat excretion. In the case of individual infants, it was seen that two infants who were excreting high quantities of starch had received high starch food for the three days prior to giving the faecal sample. An infant in the mixed fed group who had excreted 3.43 g starch / 100 g faecal wet weight at early weaning had received a high proportion of vegetables for the three days prior to giving the sample. Similarly an infant in the formula-fed group who had a value of 2.33g starch excreted / 100g faecal wet weight had consumed mashed banana on the three day prior to giving the sample which may, depending on the ripeness of the banana, contain large amounts of resistant starch (Englyst *et al.*, 1992). In contrast there were no obvious relationship between dietary fat and faecal fat.

## 8.5 CONCLUSIONS

The pilot study provided important preliminary findings on the fermentation capacity of breast-fed and formula-fed infants for simple and complex carbohydrates. A drawback was the small number of infants studied and the cross-sectional design of the experiment. However, the results allowed a larger, longitudinal study to be designed that could investigate the relative fermentation capacities for a wider range of carbohydrate substrates.

Although overall numbers recruited in each infant feeding group in the larger study did meet requirements for the power of the study, there were problems collecting

samples at each development stage. These problems included motivation of mother, production of sample at required time and mothers' returning to work. In addition recruitment of formula-fed infants was made difficult because a breast-feeding initiative which meant not as many mothers formula-fed their infants from birth. As a consequence the numbers available for longitudinal analysis were smaller than had been hoped for. However, adequate numbers were available to study the development of fermentation capacity in the three infant feeding groups.

In the pilot study, only breast-fed and formula-fed infants were investigated and compared to each other and to data from comparable adult cultures. In the main study in this thesis, a group of infants that had been given a mixture of both human and formula milk in a range of quantities were also studied. These mixed fed infants were considered important as many mothers begin breast-feeding due to the promotion of the benefits of human milk, but for several reasons may be unable to continue. Often this mixed fed group is overlooked in nutritional investigations despite the fact they are a large subgroup. The mixed fed infants in this study were a very heterogeneous group, with an infant that had been given a single bottle of formula-milk through to infants that had been breast-fed for a few days before being completely changed to formula-feeding. Increased numbers of mixed fed infants would be needed to allow comparisons between groups of infants that had been fed different amounts of breast milk and formula milk, however, it was not the aim of this investigation to compare subsets of mixed feeding infants.

Comparisons of fermentation capacity suggested that all infants were equally able to ferment the simple sugars. Raftilose™, an oligosaccharide, was also fermented equally by formula and mixed fed infants but fermentation capacity for this oligosaccharide did not increase until early weaning for breast-fed infants. In breast-fed and mixed fed infants the fermentation of pectin, one of the complex carbohydrates, did not increase until early weaning. Although there was an increase in total SCFA produced by formula-fed infants with pectin this was not significant.

The ability to ferment soyabean polysaccharide and starch was poor in all three groups of infants and had not reached adult levels even by very late weaning. However with breast-fed and mixed fed infants there was a significant increase in total SCFA produced at late weaning whereas with formula-fed infants the increase was gradual throughout the development stages.

Patterns of SCFA show the mixed fed infants to be more similar to formula-fed infants with increases in butyrate proportions whereas breast-fed infants showed increases in propionate and butyrate. This tends to suggest that at pre-and early weaning that butyrate is not as important in infants as it is in adult colonic health.

Few previous studies of fermentation capacity have been carried out in infants. Those that have measured fermentation have only measured fermentation of endogenous substrates or of added sugars (Rasmussen *et al.*, 1988; Lifschitz *et al.*, 1990).

Although in the study described in this thesis there were no differences between any groups of infants at any development stage for total faecal SCFA differences were seen in proportions of individual SCFA. Breast-fed infants had significantly lower proportions of propionate and butyrate at pre- and early weaning than formula or mixed fed infants. This was in relation to higher lactate at these stages. Mixed fed infants differed from formula-fed infants at the early stages of weaning with higher propionate and less butyrate.

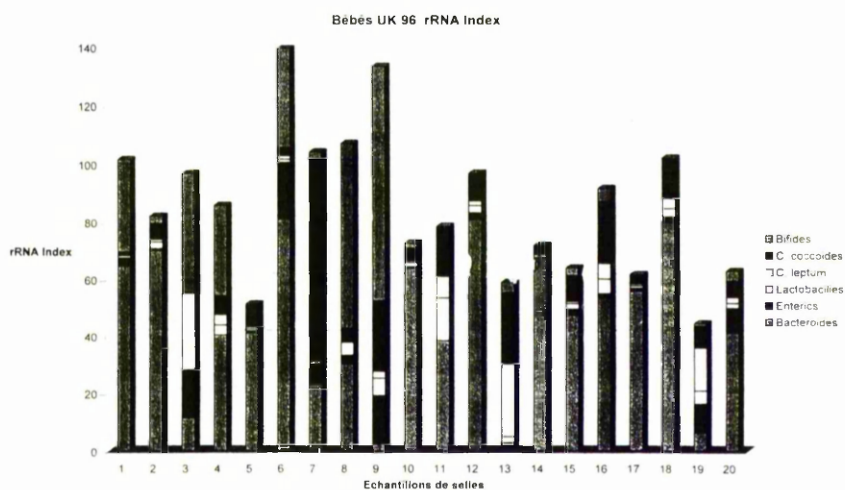
Previous studies in Denmark (Rasmussen *et al.*, 1988), Sweden (Midtvedt and Midtvedt, 1992), Estonia (Siguur *et al.*, 1993) and the U.S.A. (Lifschitz *et al.*, 1990) have measured SCFA of infants. In the studies in Sweden, Estonia and Denmark, infants investigated were breast-fed or had breast milk and glucose supplements before formula milk was substituted. These studies therefore may be of limited relevance to the UK where a large number of infants never receive human milk. In

addition, apart from the Swedish study, these were all of cross-sectional design and therefore the same infants were not studied at each age. Only the study of Lifschitz (1990) measured lactate which, is a dominant SCFA in the profile of breast-fed infants.

This study in this thesis therefore has confirmed previous knowledge of breast-fed and formula-fed infants in terms of faecal SCFA (Edwards *et al.*, 1994) and fermentation capacity (Parrett *et al.*, 1997, 1999). However, the present study represents a detailed longitudinal study of the development of fermentation capacity in infants. The differences seen in breast-fed and formula-fed infants is most likely related to the composition of the bacterial flora. Although this was beyond the scope of the present study, some of the infant faecal samples were sent to Dr J. Dore's laboratory INRA, France for analysis of rRNA with six probes for different bacterial groups. Results are shown in Figure 8.1.

Figure 8.1 rRNA analysis of a subset of faecal samples

1. Early weaned breast fed
2. Early weaned breast fed
3. Early weaned formula fed
4. 1 month breast fed
5. 7 month breast fed
6. 7 month breast fed
7. 7 month breast fed
8. 7 month breast fed
9. 7 month breast fed
10. 7 month formula fed
11. 9 month breast fed
12. 9 month breast fed
13. 9 month breast fed
14. 9 month mixed fed
15. 9 month formula fed
16. 12 month breast fed
17. 12 month breast fed
18. 12 month formula fed
19. 12 month formula fed
20. 12 month formula fed



The mixed fed infant group has been shown to be a discrete group from both the breast-fed and formula-fed infants. As the mixed fed group was so heterogeneous more studies should be carried out in larger numbers to investigate the differences of this group more thoroughly.

Development of fermentation capacity was shown to be slower in breast-fed and mixed fed infants. Whilst the fermentation capacity of formula-fed infants did not change through development stages, with complex carbohydrates it was still seen to be lower than that of adults. This suggests that development of fermentation capacity is still developing at two years of age, as has been seen with other bacterial activities (Midtvedt *et al.*, 1988). The great variability of infants within groups is also important suggesting that in some infants development of fermentation capacity is more rapid than in others and therefore is not dependent on neonatal feeding practice alone.

The slow development of fermentation capacity suggests that the colonic salvage of energy from complex carbohydrates is unlikely to contribute significantly to daily energy intake of infants. However, the faecal losses from excretion of faecal starch and fat incurred because of this slow development are miniscule and energy losses as a consequence of these should be negligible.

Continued ingestion of complex carbohydrates may alter the colonic flora of infants, inducing enzymes and altering bacterial populations which may increase the ability to ferment these substrates. If weaned onto a high-fibre diet the infants may have developed a flora better able to ferment complex carbohydrates. In the first year of life when the colonic microflora is developing it could be manipulated by diet to give future health benefits.



## 8.6 FUTURE WORK

Although the study in this thesis has given a better understanding of the development of fermentation capacity in infants there is still potential for future work. Further studies should be carried out investigating development of fermentation capacity in these three feeding groups and also other bacterial activities such as paracresol, cresol production and bacterial enzyme activity. In addition, infants from other countries should be studied to enable a fuller investigation. Feeding behaviour and weaning practice and foods are likely to vary in different parts of the world, therefore a multicentre study would provide much greater information. Within the UK more nutritional studies of mixed fed infants should be carried out, this is an important but often neglected group of infants, and have been shown in this present study to be distinct from both breast and formula infants. Dietary data should be collected to allow better correlation of diet with development of colonic flora and fermentation capacity. Finally, the infants investigated in this study should be followed up at later stages to see if differences found in this present study have any longterm effects.

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# Appendix 1

## Fermentation by gut bacteria in healthy babies

In this study we want to find out how the digestion of food changes during weaning in healthy babies. We will need fresh dirty nappies from your baby before you begin weaning (when baby is about 1 and 2 months old), just after weaning begins (as liquidised food is being introduced), when weaning is established (with solid food being given) and when the child is older (eating a full range of foods).

It is essential that we collect and process the dirty nappies within one hour of bowel movement. This is best achieved if you can telephone the department when your child has a dirty nappy. A member of staff would then come and collect it. If possible we would like to collect all the dirty nappies produced by your child over a three day period. This would mean a daily pick up of the dirty nappies which should be stored in a cool place.

We would also like to measure the weight and height of your child when we collect a dirty nappy. We would also be grateful if you could make a record of your child's food intake for the three days prior to giving samples.

Although your child will not directly benefit from this study it will provide us with important information about feeding and weaning of babies. You may withdraw from the study at any time.

### CONSENT FORM

#### Investigation of the development of colonic bacterial metabolism of the neonate during the weaning process - a longitudinal study

I,.....of.....give

my consent to the participation of ..... in the

procedures described above, which have been described to me by.....

Signed.....Date.....

Witness..... Date.....

Investigator.....Date.....

